Method development for extraction and HPLC analysis of kaempferol, chrysin and quercetin

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Dissertation accepted in fulfilment of the requirements for the degree Master of Science in Pharmaceutical Chemistry at the North-West University

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Graduation: May 2020
Student number: 25051806

ABSTRACT

Keywords: Alzheimer's disease (AD); flavonoid, chrysin, kaempferol, quercetin, high performance liquid chromatography (HPLC); thin layer chromatography (TLC); maceration; water bath extraction (WBE); ultrasonic extraction (USE); microwave assisted extraction (MAE).

Flavonoids (different phytochemical compounds found in plants acting as phytoalexins), have been found to have potent antioxidant effects. This has led into studies conducted on its effect on the body in reducing oxidative stress in different diseases as they have the potential to scavenge free reactive oxidative species that causes harm to the body, causing degeneration of cells. In the search for medicines that can delay the progression of Alzheimer's disease (AD), research into the use of flavonoids has become more popular based on the oxidative stress theory of AD. This has established its ability to reduce amyloid β plaque formation and tau protein formation in the different areas of the brain.

This study aimed to develop a HPLC method for analysis of chrysin (flavone subgroup), kaempferol and quercetin (flavonol subgroup) after extraction from selected plant material (red and yellow onions and broccoli), identifying the flavonoid compounds, estimating their quantities and drawing comparisons between different processing and storage procedures. A rough screening method using TLC was also developed.

A screening method was developed using TLC with two different mobile phases that proved adequate. This was a) toluene 80%: ethanol 20% and b) toluene 60%: ethyl acetate 30%: formic acid 10%. A HPLC method for analysis of chrysin, kaempferol and quercetin was developed and validated using the 10 mM phosphoric acid (H_3PO_4) 50%: methanol 25%: acetonitrile 25% as mobile phase with a flow rate set to 1 ml/min and the wavelength of detection set to 280 nm. A Kinetex® EVO C_{18} column (250 mm x 4.6 mm; 5 µm particle size and 100 Å pore size) was used. The plant material was processed to use whole, cut and blended plant material which were either fresh or frozen. Cooked plant material and grilled onions were also used for analysis of flavonoid content. Maceration, water bath extraction (WBE), ultrasonic extraction (USE) and microwave assisted extraction (MAE) were used to extract flavonoids from the prepared plant materials. Samples from these extractions were analysed on the HPLC. Comparisons were drawn between these different plant preparations, processing and extraction methods to establish the better method.

In this study, best extraction results were achieved by MAE, followed by USE, maceration and lastly, WBE. A rough estimation of the chrysin, kaempferol and quercetin content led to the conclusion that insufficient concentrations of these flavonoids would be obtained from such small

amounts of plant specimens used in this study. A more diverse diet with supplements of these compounds would be more beneficial to the AD patient, or any patient looking at natural treatment aimed at prevention.

ACKNOWLEDGEMENTS

To God be the glory and my saviour, Jesus Christ, who deserves all the honour for this work, as without Him, this study would not have been possible. May your Name be glorified. He was the One who carried me through when there seemed to be no light at the end of the tunnel.

My supervisors, Prof Anita Wessels and Prof Anél Petzer, thank you for your kind and caring leadership and answers that you provided. This has made the experience much more worthwhile. May God's blessing rest upon each of you.

Mom and Dad, thank you for your support during this time. Lifelong lessons aren't just learnt at home, but over as great the same distance, that's how long these lessons will stay with me as well.

To my hostel sons in whom I take pride, AJ van der Walt, Remardo Hamman and JP du Toit, thanks for always being there and always having something to share, laugh and joke about, even if it was my lack of presence. You made it worthwhile to still be here in Potch and always have someone to talk to.

Then lastly, to two remarkable friends and great men that made time in their busy schedules to sit with me during the late hours in the lab, and also helped me stay awake and vigilant when my energy was spent. Wian de Klerk and Juan Kotze, I consider you my own brothers.

To each person mentioned, the Lord bless you and keep you, the Lord make His face shine upon you, and be gracious unto you, the Lord lift up His countenance upon you and give you peace.

Proverbs 16:3

Commit thy works unto the Lord, and thy thoughts shall be established.

LIST OF ABBREVIATIONS AND ACRONYMS

A

AA	Acetic acid
Aβ	Amyloid β
ACh	Acetylcholine
AChE	Acetylcholinesterase
AChEI	Acetylcholinesterase Inhibitor
AChR	Acetylcholine Receptor
ACN	Acetonitrile
AD	Alzheimer's Disease
AECE	Apolipoprotein E Cleaving Enzyme
ALE	Advanced Lipoxidation End-products
AMP	Adenosine Monophosphate
APOE	Apolipoprotein
APOE 2	Apolipoprotein 2
APOE 3	Apolipoprotein 3
APOE 4	Apolipoprotein 4
APOE Є2	Apolipoprotein E Є2 allele
APOE Є3	Apolipoprotein E Є3 allele
APOE Є4	Apolipoprotein E Є4 allele
APP	Amyloid Precursor Protein

ATP	Adenosine Triphosphate
AUC	Area Under the Curve

B

BBB	Blood-Brain Barrier

C

C	Chrysin
C4H	Cinnamate-4-hydroxylase
Ca^{2+}	Calcium-ion
CAA	Cerebral Amyloid Angiopathy
CAT	Catalase
ChEI	Cholinesterase Inhibitors
CF	Chloroform
CHI	Chalcone Isomerase
CHS	Chalcone Synthase
CNS	Central Nervous System
CoA	Co-factor A
Cox-2	Cyclooxygenase 2
Cu^{2+}	Copper-ion
CYP1A	Cytochrome P1A

D

DAHP	3-Deoxy-D-arabino-heptulosonate-7-phosphate
DAHPS	3-Deoxy-D-arabino-heptulosonate-7-phosphate Synthase

DC	Development Chamber
DCM	Dichloromethane
DNA	Deoxyribonucleic Acid

E

E	Ethanol
EA	Ethyl Acetate
EPSP	5-Enolpyruvylshikimate-3-phosphate

F

FA	Formic Acid
FAD	Familial Alzheimer's Disease
Fe^{3+}	Iron-ion

G

GIT	Gastrointestinal Tract
GMP	Guanosine Monophosphate
GPx	Glutathione Peroxidase
GST	Glutathione S-Transferase

H

H_3PO_4	Phosphoric Acid
HIV	Human Immunodeficiency Virus
HNE	Hydroxynonenal
H_2O_2	Hydrogen Peroxide
$HO^{\cdot}$	Hydroxyl Radical

$HOO^{\cdot}$	Hydroperoxyl Radical
HPLC	High Performance Liquid Chromatography

I

ICH	International Conference on Harmonisation
IMP	Inosine Monophosphate
ISO	International Organisation for Standardisation

K

K	Kaempferol

L

LC	Locus Coeruleus
LC (a)	Liquid chromatography
LDL	Low Density Lipoprotein
LDLR	Low Density Lipoprotein Receptor
LOQ	Limit of Quantification
LPH	Lactase Phlorizin Hydrolase

M

M	Standard Mixture
MAE	Microwave Assisted Extraction
MDA	Malondialdehyde
Me	Methanol
MAO-B	Monoamine Oxidase B
MP	Mobile Phase

N

NA	Nor-adrenaline
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NFT	Neurofibrillary Tangles
Ni^{2+}	Nickel-ion
NMDA	*N*-Methyl-*D*-Aspartate
NMDAR	*N*-Methyl-*D*-Aspartate Receptor
NO	Nitrous Oxide
NP	Normal Phase
NPC	Normal Phase Chromatography
NSAID	Non-Steroid Anti-Inflammatory Drugs

O

O_2	Oxygen
$O_2^{\cdot}$	Superoxide Radical
OH	Hydroxyl group

P

P_{app}	Apparent Permeability
PAL	Phenylalanine Ammonia-lyase
PEP	Phosphoenol Pyruvate
PGE_2	Prostaglandin E_2
Phe	Phenylalanine
PS-1	Presenelin-1

PS-2	Presenelin-2
PUFA	Poly-unsaturated Fatty Acid

Q

Q	Quercetin

R

R^2	Coefficient of Determination
RDS	Rate Determining Step
R_f	Retention Factor
ROS	Reactive Oxidative Species
RP	Reverse Phase
RPC	Reverse Phase Chromatography
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
RP-TLC	Reverse Phase Thin Layer Chromatography
RSD	Relevant Standard Deviation
R_t	Retention Time

S

SD	Standard Deviation
SOD	Superoxide Dismutase
SP	Stationary Phase

T

T	Toluene
TAL	Tyrosine Ammonia-lyase

TCA	Tricarboxylic Acid
TLC	Thin Layer Chromatography
Tyr	Tyrosine

U

UHPLC	Ultrahigh Performance Liquid Chromatography
USE	Ultrasonic Extraction
USP	United States Pharmacopeia
UV	Ultraviolet

W

WBE	Water Bath Extraction

X

XDH	Xanthine Dehydrogenase
XO	Xanthine Oxidase
XOR	Xanthine Oxidoreductase

Z

Zn^{2+}	Zinc-ion

LIST OF SYMBOLS

%	percentage
°C	degrees Celsius
µg/ml	microgram per millilitre
µL	microliter
µm	micrometre
µmol/L	micromole per litre
Å	Armstrong
cm	centimetre
e^-	electron
Ɛ’	dielectric constant
Ɛ’’	dielectric loss
g	gram
g/mol	gram per mol
GHz	gigahertz
h	hora
Hz	hertz
kHz	kilohertz
mg	milligram
mg/day	milligram per day
mg/kg	milligram per kilogram

mg/L	milligram per litre
mg/ml	milligram per millilitre
MHz	megahertz
min	minute
ml	millilitre
ml	millilitre
ml/min	millilitre per minute
mm	millimetre
mM	millimolar
MPa	megapascal
nm	nanometre
s	second
tan δ	dissipation factor
V	volt
v/v	volume per volume
W	watt
ʎ	wavelength
γ	superficial tension
η	viscosity

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CHAPTER 1 INTRODUCTION

1.1 Problem statement

Alzheimer's disease (AD) is an illness that affects the elderly (with a modal age of 65 during the early stages of diagnosis) and those that have a predisposition to this illness that can be traced back to their genetics (Cummings & Cole, 2002). It is an illness that not only affects the patient, but also the family and friends of this person, leaving a toll on each of their lives (Kiecolt-Glaser *et al.*, 1987). AD has been described as being the most common cause of dementia (Cummings & Cole, 2002) and as it progresses, it reduces a patient's cognitive and physical function to the point that the person needs 24-hour (h) supervision (Jalbert *et al.*, 2008).

There is no cure for AD as it affects the brain, destroying some of the brain cells in an irreversible manner in the cortical areas as well as the medial temporal lobe (Braak & Braak, 1991). This is done by forming plaques (the aggregation and accumulation of β Amyloid (Aβ) peptides) and neurofibrillary tangles (NFT) that disrupt the signal pathways between neurons, eventually causing atrophy and loss of brain cells (Cummings, 2001; Jalbert *et al.*, 2008; McKhann *et al.*, 1984). It has also been shown that there is a decrease in neurotransmitters in the brain, especially acetylcholine (ACh), which is the focus of first-line therapy (Birks, 2006). The only treatment that is available, does not slow the progression of the illness, but with specific symptoms, mainly enhancing the patient's current cognitive and physical abilities. The current therapy that is used is Rivastigmine (Exelon®), Galantamine (Razadyne®) and Donepezil (Aricept®) which are cholinesterase inhibitors (ChEI). These drugs prevent the breakdown of ACh in the synapses during signalling (Birks, 2006; Wells *et al.*, 2015). In later stages the drug memantine (Namenda®), a N-methyl-D-aspartate (NMDA)-receptor antagonist, is used as therapy. These drugs have shown improvement in the patient's behavioural symptoms, but no significant improvement in acute agitation which is a sudden, worsened state of anxiety with mental tension and motor restlessness that occurs in the later stages of AD and other psychological conditions (Wells *et al.*, 2015). Preceding signs for tension building up include verbal signs (mutism, loud yelling and pressured speech, threats) as well as motoric signs (clenched fists, pacing, wringing of the hands, banging of objects, intense staring and physical threats). During acute agitation the patient can become assaultive towards others and themselves as well as causing property damage. Acute agitation, only after having started treatment of the underlying cause, is treated by oral or intramuscular administration of haloperidol (Serenace®), lorazepam (Ativan® and Tranqipam®), olanzapine (Zyprexa® and Olexar®), ziprasidone (Geodon®) and aripiprazole (Abilify®) (Mendelowitz, 2002; Wilson *et al.*, 2012).

It has been found by Croft (1998) and Roth *et al*. (1999) that flavonoids could have a pharmacological effect on the progression of AD by inhibiting the formation of reactive oxidative species (ROS) that destroy the brain cells. Flavonoids are present in different food sources and are thus ingested during everyday life. It is thus possible to influence the treatment and progression of this disease by changing a patient's diet and lifestyle. Among these flavonoids, the most common are chrysin (in lesser quantities), kaempferol and quercetin.

Previously, high performance liquid chromatography (HPLC) methods were developed for the analysis of flavonoids for specific plants such as onions and broccoli (Turner *et al.*, 2006). Most of these methods were developed to determine the total flavonoid content of these food sources. These methods did not focus on kaempferol, chrysin and quercetin individually. This study focuses on the development of a method for the extraction and specific analysis of these compounds.

1.2 Aims and objectives

The aim of this study is the development of a method for the extraction and specific analysis of kaempferol, quercetin and chrysin in the presence of other flavonoids from onions and broccoli using HPLC. Objectives are:

1. Development and validation of an HPLC method for the semi-quantitative analysis of the three compounds in the presence of other interfering compounds.

2. Development of an extraction method for the plant material provided.

3. Approximate quantification of the 3 components in plant extracts.

4. Development of a screening method for the particular matrices using thin layer chromatography (TLC).

1.3 Hypothesis

It is postulated that an extraction and analysis method for the separation of flavonoids chrysin, kaempferol and quercetin can be developed as based on previous methods that have been developed by Bimakr *et al*. (2011), Kim *et al*. (2002), Martino & Guyer (2004), Oniszczuk *et al*. (2016), Roldán-Marín *et al*. (2009) and Vian *et al*. (2009). The method that will be developed will focus on these three flavonoids, and not on a singular flavonoid as has been done in the previous

studies. This method will allow us to estimate the flavonoid content of different vegetables that are common in the South African diet.

1.4 Study layout

Standards of chrysin, kaempferol and quercetin will first be separated on reversed phase TLC-plates (RP-TLC).. Different mobile phases will be used as based upon previous TLC separations done by Móricz *et al.* (2018), Williams *et al.* (1997) and Panchal *et al.* (2017). A HPLC method will then be developed for the separation of the standard substances using a Hitachi Chromaster chromatographic system. The system consists of a 5410 UV-detector, an autosampler (5260) with a sample temperature controller and a solvent delivery module (5160). Different columns, wavelengths (λ) and mobile phases (MP) will be considered during method development to determine the appropriate analysis parameters.

Extractions will be made from different parts of onions and broccoli. Different extraction methods will be used, mainly maceration, ultrasonication and incubation in a water bath to find the most suitable method. Each extraction will be done on fresh samples, as well as frozen and cooked (various) vegetables. These extractions will be analysed using the HPLC method developed for the standard substances while further refinement of the method will be continuously undertaken. The refined method found to be suitable, shall be validated according to the International Conference on Harmonisation (ICH), Eurachem (Magnusson & Ornemark, 2014) and United States Pharmacopeia (USP) (Shabir, 2003).

CHAPTER 2 LITERATURE STUDY

2.1 General background

Polyphenols are applied for different industrial uses such as processing of cosmetics, additives in the food industry, paper, paints and tanning agents. They are synthesised in plants via the shikimate- and acetate pathways as products of secondary metabolism (Bravo, 1998; Ross & Kasum, 2002). Flavonoids, which are plant polyphenols, have been shown to be a low risk alternative for treatment of various diseases. The flavonoids' pleiotropic characteristics are responsible for the different pharmacological mechanisms of action leading to various uses and pharmacological studies (Karuppagounder *et al.*, 2016). Flavonoids are ingested in a person's normal diet and are estimated at 200-350 mg/day (Johannot & Somerset, 2006) The flavonoid content of different foods, including intra-species, can vary because of climate, cultivar, farming practices, geography, processing and storage conditions (Amiot *et al.*, 1995; Häkkinen *et al.*, 2000; Patil *et al.*, 1995). Different flavonoids could have various effects on a person's health as it has been seen that different types of food produce are eaten at higher levels in certain age groups; that later on declines or is replaced by a different food source that is rich in the same compound affecting disease patterns in various ages (Johannot & Somerset, 2006). Flavonoids have been called "vitamin P" as their level of importance has been made known. Later research led to the dismissal of the name "vitamin P", but flavonoids are still under constant study (Kuo, 1997). This is because it is of importance to know what effect a person's diet has on chronic and genetic diseases as this can help in prevention tactics as well as dietary treatment of different diseases. It has been found that low consumption of fruits and vegetables increased the likelihood of cancer twofold compared to subjects who consume high amounts of fruit and vegetables (Boyer & Liu, 2004).

2.2 Flavonoids

This chemical class of compounds can be found in plants as aglycones, but are usually in a glycosylated form when it is produced. Even after ingestion, they are more commonly found in their glycosylated forms (Day *et al.*, 2000). The glycosides can be either in the form of mono-, di- or oligosaccharides, or sugar residues of galactose, rhamnose, xylose, arabinose, galatoronic or glucoronic acids (Bravo, 1998). This glucose attachment causes the flavonoid molecule to be more easily water soluble (Ross & Kasum, 2002). The glucose-moiety enlarges the molecule, making it harder to be absorbed, where the aglycone form is more easily absorbable. A fruit type can have 6 to 7 different glycosides present (Manach *et al.*, 1996).

Being a phytochemical, flavonoids have different characteristics that make them unique. They serve as a light screen against damaging ultraviolet (UV) rays in new leaves, they have antioxidant activities, they can inhibit certain enzymes, they serve as precursors of certain toxic substances, they are resistant to pathogens, they are photosensitising compounds, they are energy transferring compounds, they are active in the control of plant growth and development in combination with hormones and they are heat stable (Yao *et al.*, 2004).

2.2.1 Chemical background

The flavonoid molecule itself is a planar molecule. As previously stated, flavonoids are synthethised via the shikimate (a metabolic pathway found in plants, fungi, bacteria, algae and parasites which name is derived from the metabolite shikimic acid) and acetate pathways in plant cells (Tzin *et al.*, 2012; Weaver & Herrmann, 1997). Phenylalanine (Phe), tyrosine (Tyr) and malonate are formed in plants from the precursor chorismate. Flavonoids themselves are synthesised in plants from these aromatic amino acids (which are precursor forms for production of flavonoids, lignin's, tannins and coumarins) via the shikimate pathway (Croteau *et al.*, 2000; Stalikas, 2007; Weaver & Herrmann, 1997; Yao *et al.*, 2004). The 4-oxo-flavonoid is synthesised from a common intermediate, namely tetrahydroxychalcone, which is the result of three malonyl-CoA units that are condensed with a derivative from hydroxycinnamic acid via the acetate pathway later on in the sequence (Manach *et al.*, 1996). These compounds are responsible for the bright colours in the flowers parts of plants (Stalikas, 2007; Yao *et al.*, 2004).

The main compound is based upon a C_6-C_3-C_6 flavone skeleton (Biesaga, 2011). The C_3 carbon bridge found between the two phenyl groups is usually cyclised with oxygen as shown in Figure 2.1:

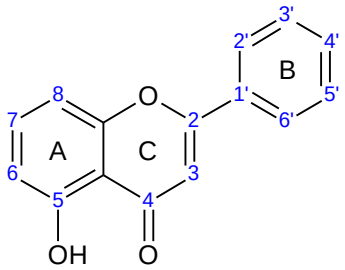


Figure 2.1: The basic chemical structure of a flavonoid molecule. Aromatic ring A is produced from malonyl-CoA and ring B from the shikimate pathway.

Figure 2.1 shows that rings A and B are phenyl rings and that ring C is a pyran ring. The A-ring is biogenetically formed from malonyl-CoA molecules via the acetate pathway, which leads to

hydroxylation of the 5 and 7 positions, while the B ring is formed via the shikimate pathway with a usual hydroxylation pattern of the 4'-, 3',4'- or 3',4',5' positions (Bravo, 1998; Croft, 1998; Ross & Kasum, 2002). The synthesis routes responsible for the production of flavonoids are shown in figure 2.2, being the shikimate pathway, figure 2.3 showing production of the aromatic amino acids Phe and Tyr, figure 2.4 showing carboxylation of acetyl-CoA from the acetate pathway and figure 2.5 showing the formation of the flavonoid itself:

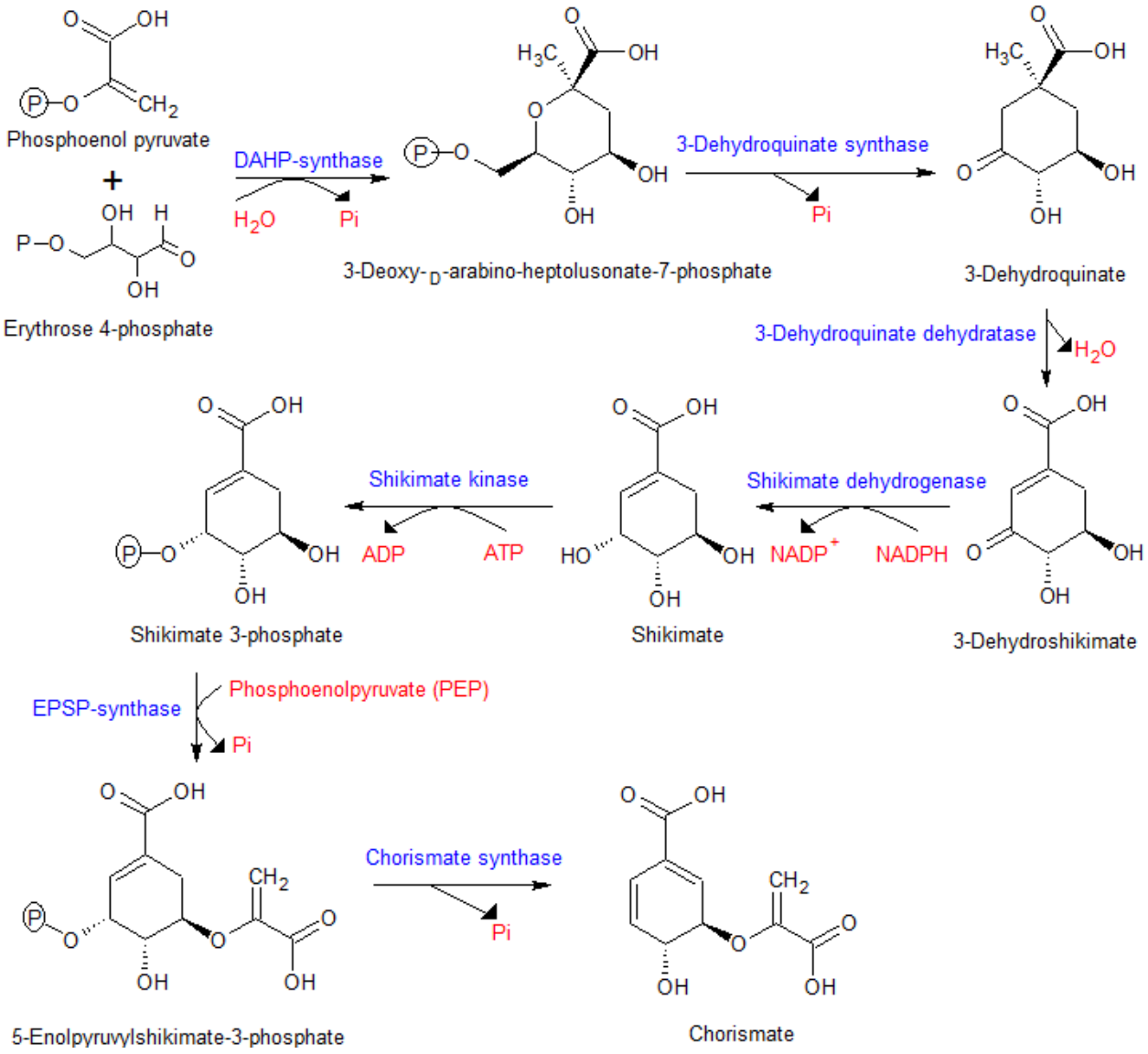


Figure 2.2: The shikimate biosynthetic pathway in plants (Akeroyd & Synge, 1992; Tzin *et al.*, 2012; Weaver & Herrmann, 1997).

Enzymes used in the shikimate pathway are encoded by wild type genes in higher plants (plants having complex or advanced characteristics such as vascular plants with flowers) (Akeroyd & Synge, 1992; Tzin *et al.*, 2012; Weaver & Herrmann, 1997). Biosynthesis in the shikimate process follows seven steps (Weaver & Herrmann, 1997). In step 1 phosphoenol pyruvate (PEP) and

erythrose-4-phosphate becomes condensed by 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAHPS), forming 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) (Weaver & Herrmann, 1997). Water is used, causing the release of a phosphate-atom. In step 2 the oxygen in the 7-position of the exocyclic ring is exchanged with carbon via 3-dehydroquinate synthase forming 3-dehydroquinate, releasing the remaining phosphate group from DAHP. During step 3, a water molecule is released from 3-dehydroquinate via 3-dehydroquinate dehydratase forming 3-dehydroshikimate. In step 4 the H-atom from nicotinamide adenine dinucleotide phosphate (NADP) in the form of NADPH is released to form shikimate with the by-product of $NADP^+$ via shikimate dehydrogenase. In step 5 the enzyme shikimate kinase uses one adenosine triphosphate (ATP) molecule of the plant, adding a phosphate group to shikimate, forming shikimate-3-phosphate. During step 6 another PEP molecule is added to shikimate-3-phosphate to form 5-enolpyruvylshikimate-3-phosphate (EPSP) using the EPSP-synthase enzyme of the plant. A phosphate group is released from PEP. In step 7, the final step, chorismate synthase releases the remaining phosphate group from EPSP forming chorismate (Tzin *et al.*, 2012; Weaver & Herrmann, 1997)**.**

Chorismate serves as the precursor for the biosynthesis of Phe and Tyr in the plant (Weaver & Herrmann, 1997). Chorismate mutase converts chorismate to prephenate. The complete biosynthesis route of Phe in plants is not completely known. Evidence from recent studies suggest that Phe follows the arogenate intermediate metabolic route. To form arogenate, the enzyme prephenate aminotransferase is used. Another suggestion is the use of phenylpyruvate metabolite for the formation of Phe. Tyr-biosynthesis follows the first two steps of Phe-biosynthesis producing arogenate. Arogenate dehydrogenase is the final step that converts arogenate to tyrosine. These biosynthetic pathways occur in the plastid (a double membrane organelle in the cytoplasm of plants that contains proteins, pigments, oil and starch) of the plant (Tzin *et al.*, 2012; Weaver & Herrmann, 1997). Figure 2.3 shows the suggested biosynthesis routes from chorismate to Phe and Tyr:

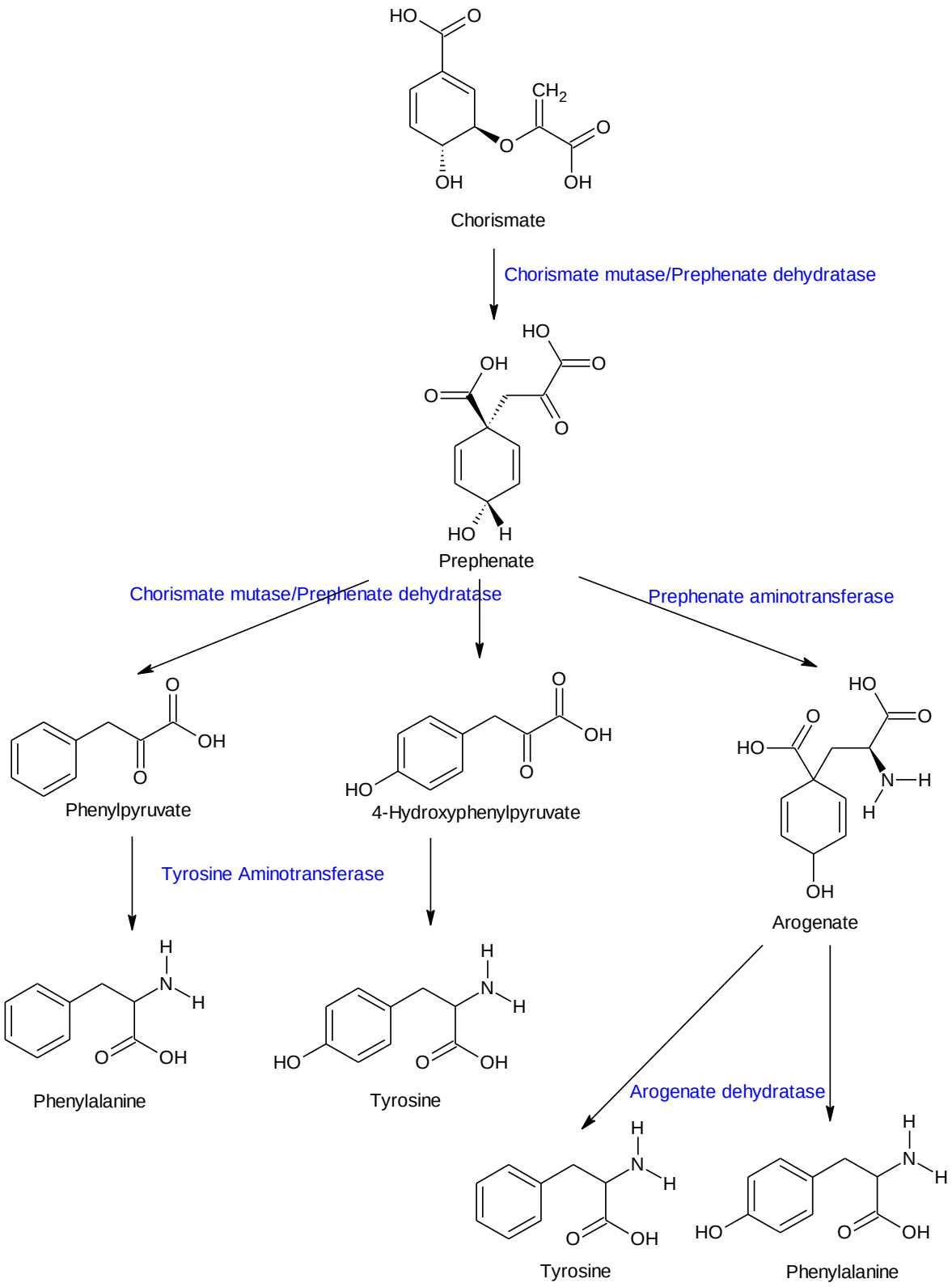


Figure 2.3: **The biosynthetic formation of Phenylalanine and Tyrosine from Chorismate obtained from the Shikimate pathway in the plastid of plants (Weaver & Herrmann, 1997).**

The acetate pathway provides three malonyl-CoA molecules that is incorporated in the biosynthesis of flavonoids. This occurs through a carboxylation reaction (Croteau *et al.*, 2000). Figure 2.4 shows the activity of acetyl-CoA carboxylase:

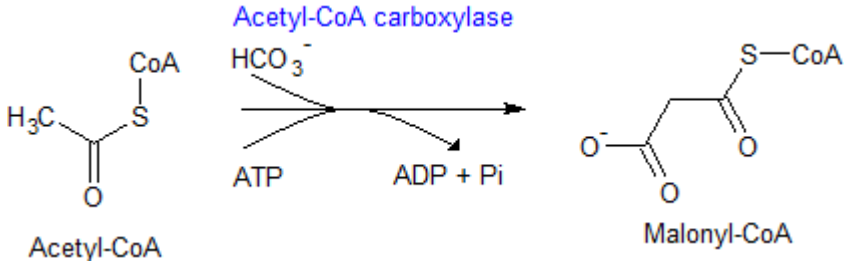


Figure 2.4: Production of malonyl-CoA in the acetate pathway to be incorporated in flavonoid production (Karpe & Broom, 2014; Croteau *et al.*, 2000).

In figure 2.4, the acetyl-CoA is obtained from the tricarboxylic acid (TCA) cycle. Acetyl-CoA is converted to malonyl-CoA via the enzyme acetyl-CoA carboxylase in the presence of bicarbonate and an expendable ATP-molecule (Karpe & Broom, 2014; Croteau *et al.*, 2000).

In figure 2.5 the final incorporation of Phe and Tyr enzymes from the shikimate pathway and incorporation of malonyl-CoA molecules from the acetate pathway is shown:

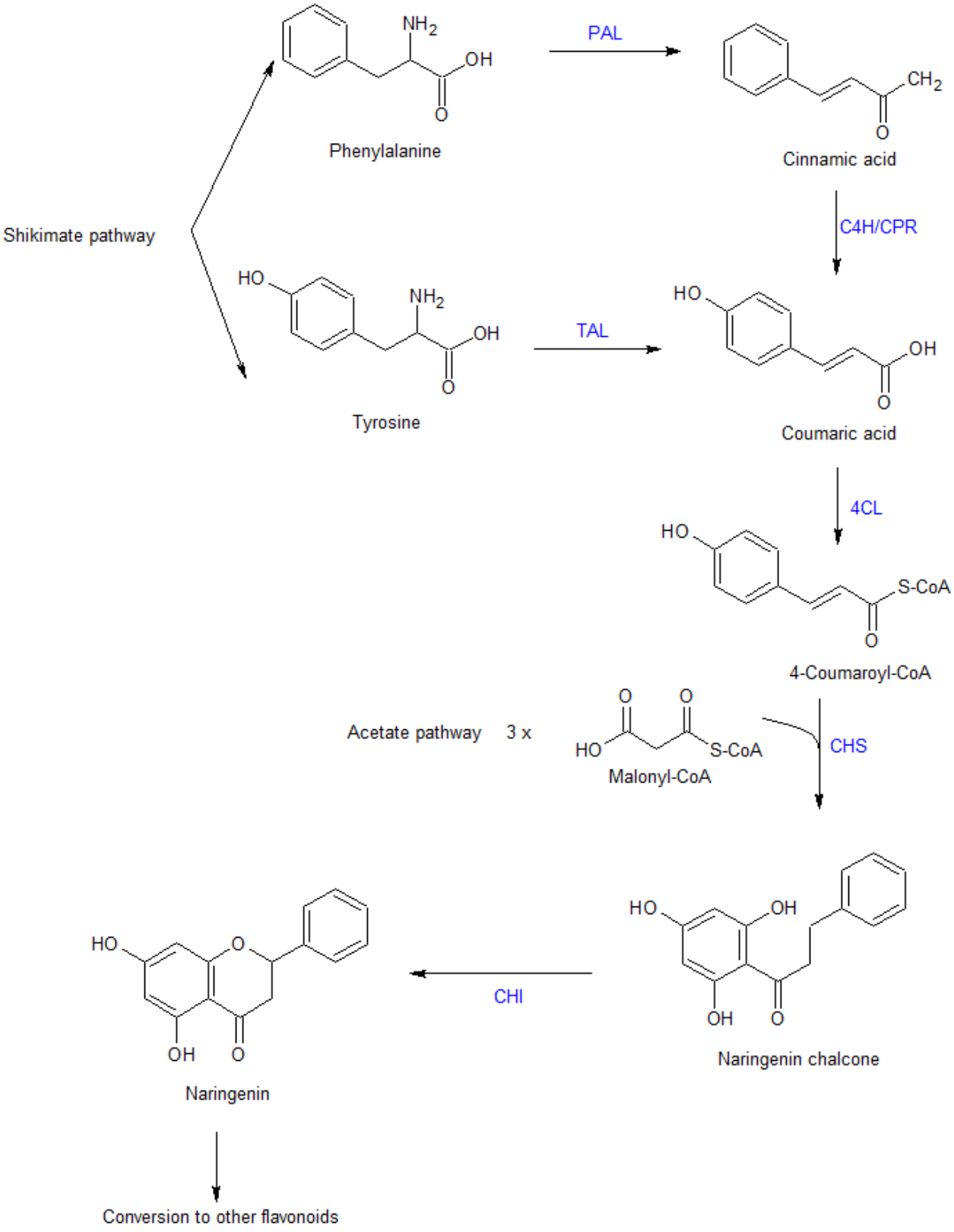


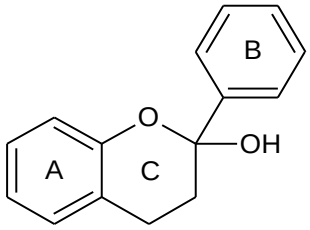
Figure 2.5: The production of flavonoids from the shikimate and acetate pathways (Croteau *et al.*, 2000; Wang *et al.*, 2011).

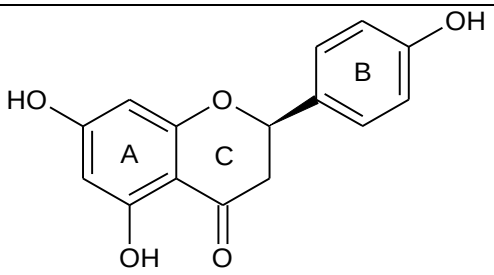
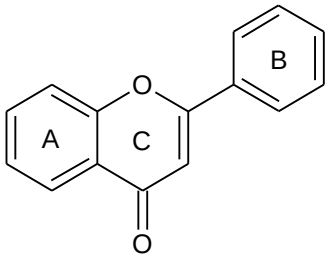
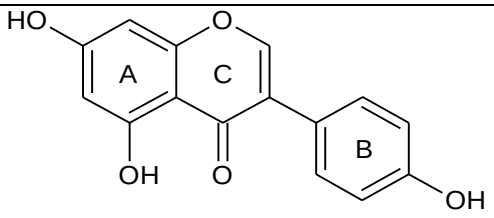
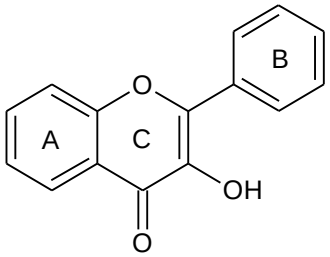
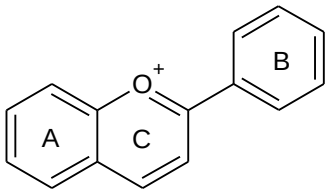
In figure 2.5, Phe is converted to cinnamic acid via phenylalanine ammonia-lyase (PAL). This is further converted to coumaric acid by the enzyme cinnamate-4-hydroxylase (C4H). Tyr is directly converted to coumaric acid by the enzyme Tyrosine ammonia-lyase (TAL). The next step is conversion of coumaric acid to coumaroyl-CoA by the enzyme 4-cumarato-CoA. For the final production of a flavonoid molecule (in this case naringenin), one p-coumaroyl-CoA molecule and three malonyl-CoA molecules from the acetate pathway are needed as starting substrates. When these molecules react under the influence of chalcone synthase (CHS), naringenin chalcone is formed. The final step is when chalcone isomerase (CHI) is used to form the flavonoid naringenin (Croteau *et al.*, 2000; Wang *et al.*, 2011).

Considering the different substitutions that can occur on all three of these rings, different subclasses (six major subclasses) of the flavonoids can be distinguished. Each subclass is characterised by the presence of hydroxyl (OH) groups on the phenyl rings (as can be seen in table 1), and distinct hydroxylation and conjugation patterns of the C-ring. The conjugation of the double bonds of the flavonoid structure allows for electron delocalisation. Their solubility is dependent on their polarity and chemical structure. This makes it possible for them to be linked to cell wall components such as lignins and polysaccharides. Solubilisation in alkaline conditions are made possible by the ester linkages of flavonoids (Bravo, 1998). Substitution with OH-groups explains the antioxidant and chelating properties of flavonoids (Corcoran *et al.*, 2012).

The six major subclasses of flavonoids are flavan-3-ols (flavanols), flavanones, flavones, isoflavones, flavonols and anthocyanins. Table 2.1 shows the differences between the different subclasses.

Table 2.1: The different subgroups of flavonoids.

The different subgroups of flavonoids:			
Subgroup:	**Chemical Structure**	**Molecular Formula**	**Molecular Mass**
Flavan-3-ol		$C_{15}H_{14}O_2$	226.275 g/mol

Flavanone		$C_{15}H_{12}O_2$	224.259 g/mol
Flavone		$C_{15}H_{10}O_2$	222.243 g/mol
Isoflavone		$C_{15}H_{10}O_2$	222.243 g/mol
Flavonol		$C_{15}H_{10}O_3$	238.242 g/mol
Anthocyanin		$C_{15}H_{11}O^+$	207.252 g/mol

Among these classes, flavonols are the most abundant in different plants and has a daily intake of 20 mg/day. This specific class is characterised by having a non-phenolic OH-group at position 3 of the base structure. More than 380 flavonol glucosides have been described, of which 200 are kaempferol and quercetin glucosides (Bravo, 1998; Manach *et al.*, 1996). The three major flavonols are quercetin, which is ingested at about 10 mg/day, myricetin and kaempferol (Yao *et al.*, 2004). They have an additional OH-group in positions 5 and 7 (Manach *et al.*, 1996). It has

been seen that processing of flavonol containing foods reduces the amounts of all three chemical compounds contents within the food source, with the biggest loss being myricetin and kaempferol (Häkkinen *et al.*, 2000).

2.2.2 Specific flavonoids

2.2.2.1 Chrysin

Chrysin (5,7-dihydroxy-2-phenylchromen-4-one) is a flavonoid derivative classified under the flavone subgroup and is naturally occurring in different plants, fruits and vegetables, such as the blue passion flower and products from plants such as propolis and honey (Hadjmohammadi & Nazari, 2010; Tomás-Barberán *et al.*, 2001). It has the same basic chemical structure as a normal flavonoid compound, except for its characteristic absence of hydroxyl groups on the 5th and 7th positions of the C-ring, making it more stable and lipid soluble than other flavonoids. The B-ring does not have any OH-groups and lack oxygenation when compared to other flavonoids (Nabavi *et al.*, 2015). This means that chrysin is more hydrophobic in comparison to other flavonoids (Walle *et al.*, 1999). Chrysin is obtained by biosynthesis from Phe (Mani & Natesan, 2018). It is proposed that a daily dose of 0.5-3 g chrysin (Mani & Natesan, 2018) can increase a person's testosterone level as it is an aromatase inhibitor, preventing the conversion of testosterone and androstenedione to estradiol and estrone, respectively (Dean, 2004). It is also an anti-inflammatory molecule that can inhibit prostaglandin E_2 (PGE_2) and cyclooxygenase 2 (Cox-2). In models of latent infection by Human Immuno-deficiency Virus (HIV), chrysin was shown to be a potent inhibitor of its activation by inhibiting HIV-1 transcription and casein kinase II (Critchfield *et al.*, 1997). Figure 2.6 shows the chemical structure of chrysin:

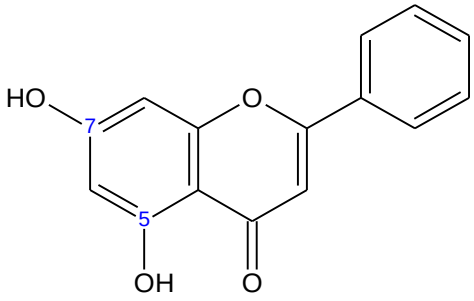
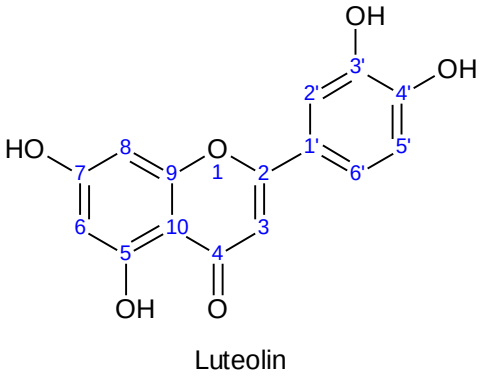


Figure 2.6: Chemical structure of chrysin.

When comparing chrysin's antioxidant activity to other flavonoids, it can be seen that hydroxylation on the B-ring is not essential, as the chemical still showed hydroxyl radical scavenging activity when it was compared to luteolin that has OH-groups at the 3',4'-positions

and a double bond between carbons at positions 2 and 3 (Harris *et al.*, 2006). In figure 2.7 the structure for luteolin is shown:



Luteolin

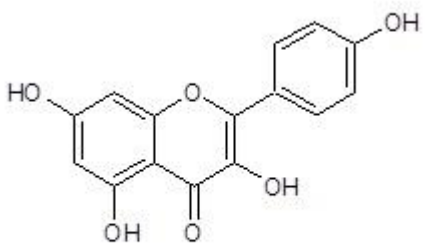
Figure 2.7: The structure for luteolin (Leopoldini *et al*., 2004).

In figure 2.7 it can be seen that the structure for luteolin is unique as a flavonoid as it shows hydroxyl groups in the 3'4'-positions and a double bond between the carbons of position 2 and 3 (Leopoldini *et al*., 2004).

2.2.2.2 Kaempferol

Kaempferol [3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one] is a yellow crystalline solid (National Center for Biotechnology, 2018a) that is found in plants as shown in figure 2.8 (Calderon-Montano *et al.*, 2011). It is synthesised in plants by condensing 4-coumaroyl-CoA with three molecules of malonyl-CoA to give tetrahydroxychalcone. Naringenin chalcone is synthesised by CHS, after which the naringenin flavanone is obtained by closing the C3 ring through the action of CHI. An OH-group is attached at the C3 position through flavanone 3-dioxygenase enzyme creating dihydrokaempferol. Kaempferol itself is the final product after the flavonol synthase enzyme introduces the double bond at the C2-C3 position (Calderon-Montano *et al.*, 2011; Winkel-Shirley, 2001).

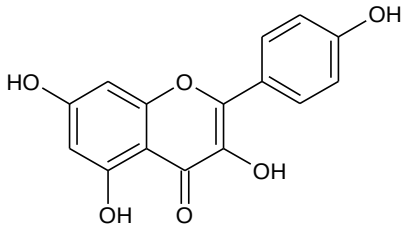
The final chemical structure of kaempferol as synthesised in plants is shown in Figure 2.8:



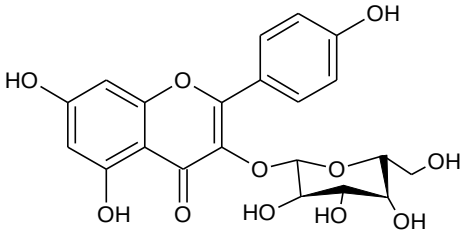
Kaempferol

Figure 2.8: The chemical structure of Kaempferol (Calderon-Montano *et al.*, 2011; Winkel-Shirley, 2001).

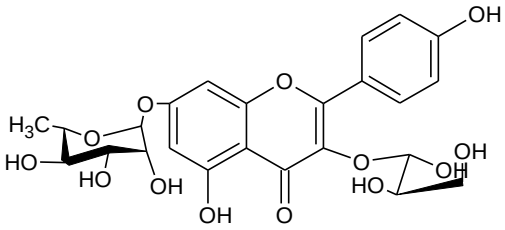
Kaempferol is slightly soluble in water, but exhibits good solubility in ethanol and diethyl ether (National Center for Biotechnology, 2018a). Kaempferol can also be bound to sugars in nature, which includes glucose, rhamnose, rutinose and galactose, that form glycosides (Calderon-Montano *et al.*, 2011). Some of these sugar bound kaempferol moieties include kaempferol-3-O-glucoside (astragalin) which is common in nature; and other less common forms, as the biosynthesis of these other glycosidic forms are more restricted as the enzymes necessary to form them are not common in all plants. They are kaempferol-3-*O*-neohesperidoside, kaempferol-3,7-dirhamnoside (kaempferitrin), kaempferol-3-*O*-(6''-E-p-coumaroyl)-glucoside (tiliroside), kaempferol-3-*O*-robinoside-7-*O*-rhamnoside (robinin), kaempferol-3-*O*-(3'',4''-di-o-acetyl)-rhamnoside and kaempferol-3-(p-coumaroyl)-triglucoside. When they become hydrolysed by the bacteria in the gut, the final aglycone product is kaempferol (Calderon-Montano *et al.*, 2011; De Melo *et al.*, 2009; Macdonald *et al.*, 1983). Figure 2.9 shows kaempferol and some of its different glycosides:

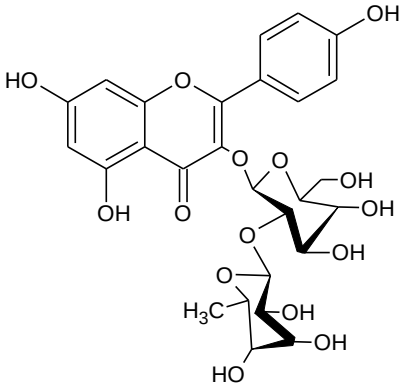


Kaempferol

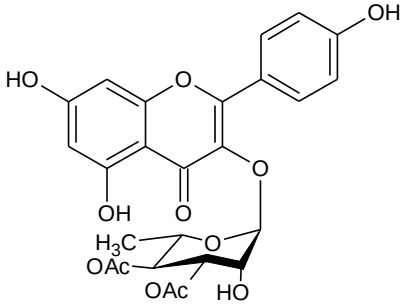


Kaempferol-3-*O*-glucoside (Astragalin)

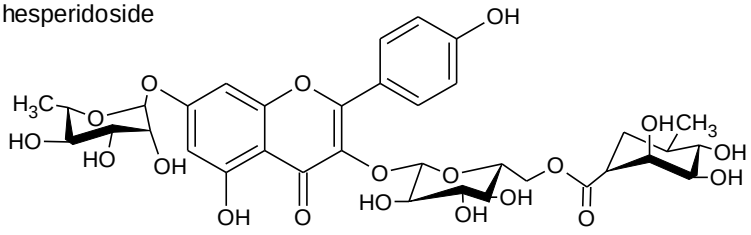




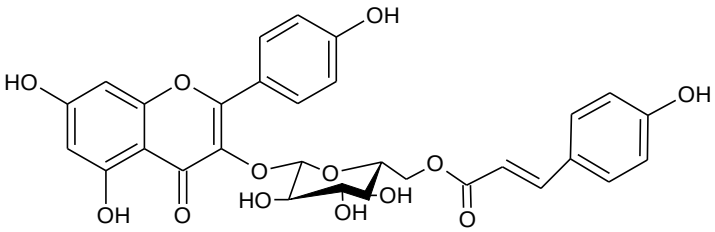
Kaempferol-3-*O*-neohesperidoside



Kaempferol-3-*O*-(3,4-di-o-acetyl)-rhamnoside



Kaempferol-3-*O*-robinoside-7-*O*-rhamnoside (Robinin)



Kaempferol-3-*O*-(6-E-p-coumaroyl)-glucoside (Tiliroside)

Figure 2.9: Kaempferol and some of its sugar moieties found in different plants (Calderon-Montano *et al*., 2011).

The most common edible products in which kaempferol can be found are onions, kale, leeks and blueberries (Corcoran *et al.*, 2012). It has also been shown that kaempferol is a transition-metal chelating molecule that lessens oxidative damage (Mladěnka *et al.*, 2011).

In plants, kaempferol has phytoalexin properties owing to its antimicrobial activity in the body (Harborne & Williams, 2000). The activities of certain antibiotics are enhanced synergistically against bacteria that has become resistant when kaempferol is used in conjunction with the antibiotic (Otsuka *et al.*, 2008). Kaempferol was shown to be an active anti-inflammatory compound found in natural food sources and also has antinociceptive (pain relieving) effects by inhibiting prostaglandin synthesis (De Melo *et al.*, 2009). Kaempferol ingestion is also associated with an inverse risk for lung- (Garcia-Closas *et al.*, 1998), gastric- (Garcia-Closas *et al.*, 1999), pancreatic- (Nöthlings *et al.*, 2007) and epithelial ovarian cancer (Gates *et al.*, 2007). It is one of the more potent antioxidant scavenging molecules for hydroxyl radicals generated by the Fenton-reaction, as well as for the peroxynitrite radical.

2.2.2.3 Quercetin

Quercetin [2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one], a yellow powder (National Center for Biotechnology, 2018b), is one of the more abundant flavonoid compounds in onions (Sharma & Lee, 2016), apples and teas (Alluis & Dangles, 2001). Onions (*Allium cepa* L.) themselves are the biggest source of quercetin in the diet as it contains approximately 300 mg/kg quercetin in fresh onions (Hertog *et al.*, 1993). Natural sources such as red leaf lettuce (*Latuca sativa* L.) and asparagus (*Asparagus officianalis* L.) contain high amounts of quercetin, while tomatoes, broccoli, peas and green peppers contain lesser amounts (Costa *et al.*, 2016). Figure 2.10 shows the chemical structure of quercetin:

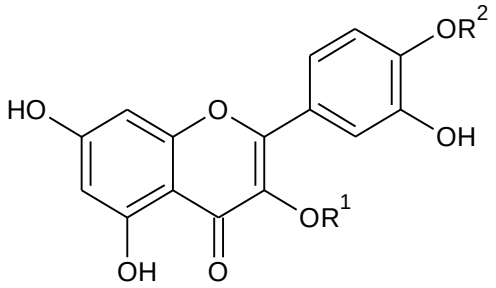


Figure 2.10: The chemical structure of quercetin.

In food sources, quercetin is present as quercetin glycosides that can be converted into the aglycone form by bacterial hydrolysis (Macdonald *et al.*, 1983). These constitute a quercetin

molecule bound to a sugar moiety forming quercetin-3-*O*-*β*-rutinoside (IV), quercetin-4'-*O*-*β*-D-glucoside (II) and quercetin-3-*O*-*β*-D-galactoside etc. The physicochemical properties of the chemical are influenced by the sugar moiety in the body, affecting quercetin's absorption and bioavailability (Day *et al.*, 2003; Hollman *et al.*, 1997). Thus, it has to be ensured that when testing total quercetin in the body, it is exactly documented which chemical forms are present in the different types of food sources being used. Figure 2.11 shows quercetin and some of its glycoside forms that can be found in natural resources. Hollman *et al.* (1997) found that the mean peak level of quercetin absorption from onions was reached after 0.5 h.

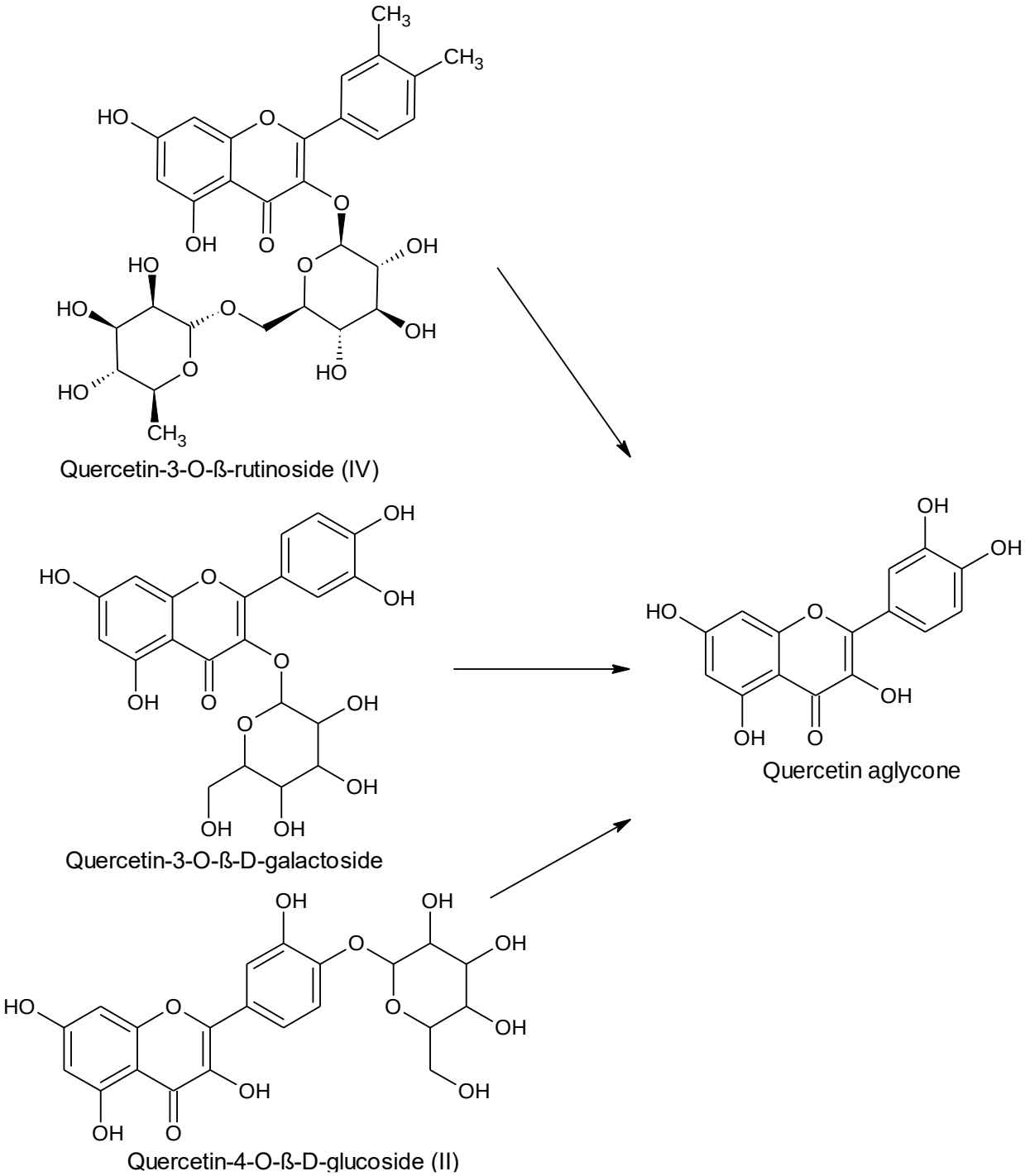


Figure 2.11: Quercetin-3-O-β-rutinoside (IV), quercetin-4'-O-β-D-glucoside (II) and quercetin-3-O-β-D-galactoside structures as well as the quercetin aglycone structure.

Table 2.2 shows the different substitutions of quercetin and its glycosides (Lee & Mitchell, 2012):

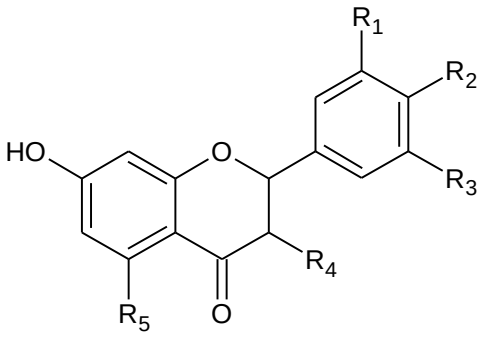


Table 2.2: The different substitutions of quercetin and its glycosides.

Quercetin Glycoside name	R_1	R_2	R_3	R_4	R_5	MW
Quercetin-3,4'-*O*-diglucoside*	OH	O-glucose	H	O-glucose	OH	626.5
Quercetin-3-*O*-rutinoside	OH	OH	H	O-rutinoside	OH	610.5
Quercetin-3-*O*-galactoside	OH	OH	H	O-galactoside	OH	464.4
Quercetin-3-*O*-glucoside	OH	OH	H	O-glucoside	OH	464.4
Quercetin-3-*O*-rhamnoside	OH	OH	H	O-rhamnoside	OH	448.4
Quercetin-4'-*O*-glucoside*	OH	O-glucose	H	OH	OH	464.4
Quercetin aglycone	OH	OH	H	OH	OH	302.2
Isorhamnetin aglycone	OCH_3	OH	H	OH	OH	316.3

*Glucocidic forms of quercetin found in onions, all others are found in apples (Boyer & Liu, 2004).

Up until age 16-18 years, apples are the most important source of quercetin in a person's diet, after which it is replaced by onions as shown in a study done in an Australian population in 2006 (Johannot & Somerset, 2006). Special emphasis should be placed on its antioxidative quality, as it reacts with ROS causing a decrease in the total amount of reactive phenoxy radicals (Yang *et al.*, 2014). When quercetin becomes unstable, it functions as a pro-oxidant that is responsible for the deterioration of plant cells, shortening its lifetime, which contrasts its antioxidative function.

Chemical properties of quercetin include good water solubility, a high degree of polarity and diamagnetic properties (Mendoza-Wilson & Glossman-Mitnik, 2005). Quercetin is one of the more potent transition-metal chelating molecules that lessens oxidative damage (Mladěnka *et al.*, 2011). It is suggested that quercetin can be used as a more natural treatment for inflammatory skin diseases and neurodegenerative neurological diseases as it can lessen the amount of neurotoxic chemicals, as has been shown in clinical trials and animal studies (Karuppagounder *et al.*, 2016; Yang *et al.*, 2014). It is also a xanthine oxidase (XO) inhibitor (Day *et al.*, 2000).

2.2.3 Pharmacokinetics

2.2.3.1 Introduction

In vivo, quercetin and kaempferol have shown poor bioavailability due to their poor absorption and rapid metabolism (Arts *et al.*, 2004; Karuppagounder *et al.*, 2016), which follow a biphasic curve (Erlund *et al.*, 2000; Hollmann, 1996). Absorption occurs via the gastrointestinal tract (GIT) in the stomach and small intestines (Lee & Mitchell, 2012). Chrysin should have a better absorption as it penetrates the intestinal wall more easily because of its lipid solubility, but this is hampered by its presystemic metabolism (Lee & Mitchell, 2012). It has been found that chrysin has a much better apparent permeability (P_{app}) than quercetin (being almost 20% better than quercetins), as it was almost completely absorbed in a transport study done with Caco-2 cells (Walle *et al.*, 1999).

2.2.3.2 First pass effect

Quercetin glucosides and aglycone obtained from onions have shown a greater absorption than those obtained from apples, and thus has improved availability from the small intestines (Boyer & Liu, 2004). Compared to absorption of pure quercetin-3-rutinoside which is mainly found in tea, the glucoside forms of quercetin found in onions showed rapid and better absorption. For the rutinoside to show effective absorption, the rutinoside needs to be hydrolysed by intestinal flora (Erlund *et al.*, 2000; Hollman *et al.*, 1997).Quercetin-glucosides do show partial absorption in the ileum, which could be due to the intestinal flora in this area's glycosidase-activity (Bokkenheuser *et al.*, 1987). The quercetin glucosides become deglycosylated to yield quercetin aglycone by hydrolysis by microorganisms in the distal ileum and caecum of the colon before passive absorption can occur, making them essential (Manach *et al.*, 1996; Walgren *et al.*, 1998). Walgren *et al*. (1998) suggested that if the 3'-position has a sugar moiety attached, absorption is promoted, while in the 4'-position, it is prevented. This could also be due to sugar carriers that are found in the intestine (Hollman *et al.*, 1995).

The *β*-glycosidic bond formed between the flavonol and glycoside is resistant to hydrolysis by pancreatic cells as the human body does not form its own enzymes with hydrolysis action. The intestinal wall of the small intestine does not secrete enzymes that can break these *β*-glycosidic bonds (Hollman *et al.*, 1995). This caused the belief that absorption of flavonoids into the body is completely hampered (Hollman *et al.*, 1997). Later it was found that lactase phlorizin hydrolase

(LPH) is an important determinant of absorption found in the brush border epithelium of the intestines responsible for quercetin's rapid absorption of the aglycone as it also hydrolyses glucosides (Hollman *et al.*, 1995; Kawabata *et al.*, 2015; Németh *et al.*, 2003). Diffusion over the brush border epithelium will be less if the *β*-glucosidase activity is low, as the cleavage of the sugar moieties would be reduced in quercetin glucosides (Day *et al.*, 2003). The sugar moiety does not have antioxidant activity and needs to be hydrolysed to the aglycone to have full antioxidant activity in the body (Ioku *et al.*, 1995; Ratty & Das, 1988; Ross & Kasum, 2002). It should be considered that the sugar moieties prevent apical efflux of the flavonoid (Day *et al.*, 2003). Absorption studies of quercetin are complicated by degradation by the flora of the colon as these microorganisms, *Bacteroides distatonis*, *B. uniformis* and *B. ovatus* which possess *β*-glycosidases, not only hydrolyses quercetin glycosides into the aglycone, but can lead to the opening of the heterocycle ring of the flavone, converting it into simple phenolic compounds that is excreted in urine (Bravo, 1998; Hollman *et al.*, 1995; Manach *et al.*, 1996; Spencer *et al.*, 2004). These bacteria form flavonoid-glycoside-hydrolysing enzymes independent of the presence of these flavonoids in their vicinity (Bokkenheuser *et al.*, 1987). During a 24 h trial, there was still quercetin present in the plasma being tested, suggesting that quercetin has a long half-life (Hollman *et al.*, 1997).

Quercetin can be seen in plasma approximately 30 minutes after ingestion, as is common with compounds that undergo passive diffusion in the small intestine (Arts *et al.*, 2004; Hollman *et al.*, 1997; Lee & Mitchell, 2012). Quercetin metabolites have a high affinity for albumin, becoming tightly bound to the albumin fraction of blood (Manach *et al.*, 1995). This is because quercetin is a polydentate molecule with many different available binding sites because of the many hydrophobic reaction sites (that first form hydrophobic bindings), that become reinforced by hydrogen bindings at the phenolic areas (Manach *et al.*, 1995). Albumin is also responsible for delivering quercetin to the liver for metabolism (Manach *et al.*, 1995).

2.2.3.3 Hepatic metabolism

It should be considered that quercetin, chrysin, kaempferol and other flavonoids undergo extensive metabolism through glucuronidation and sulphation, because of the high amount of dietary exposure to these compounds (Galijatovic *et al.*, 1999; Walle *et al.*, 1999). When quercetin and chrysin are absorbed into the enterohepatic pathway, it undergoes conjugation reactions in the small intestine and liver (Walle *et al.*, 1999). The place of conjugation on the chemical structure is significant as this influences the *in vivo* function of the molecule. The OH-groups that are mostly glucuronidated are at the 3' > 7 > and 5- positions on the catechol ring (Day *et al.*, 2000). In other

studies done, it has been seen that chrysin does not conjugate at the 5-position, as the more acidic 7-position is more accessible (Galijatovic *et al.*, 1999). Some studies have found that interconversion between kaempferol and quercetin do occur if kaempferol is hydrolysed by cytochrome P1A (CYP1A) at the 3'-position (Breinholt *et al.*, 2002). *O*-Methylation does also occur during conjugation reactions, which can be excreted in the bile, owing to the biphasic curve mentioned earlier, at which point it is distributed to the brain and muscles (Arts *et al.*, 2004; Erlund *et al.*, 2000; Kawabata *et al.*, 2015).

2.2.3.4 Bioavailability in the brain

The long half-life of quercetin suggests that subsequent ingestion of edible products containing the compound could cause an accumulation of quercetin in the plasma (Hollman *et al.*, 1997; Hollmann, 1996; Ross & Kasum, 2002). Quercetin has shown to penetrate the blood-brain barrier (BBB) (Ho *et al.*, 2012), but is dependent on lipophilicity, suggesting that BBB permeability is possible for flavonoids and their metabolites. Some of the O-methylated flavonoids, like 3'-O-methyl quercetin (isorhamnetin), have shown greater uptake than their aglycone counterparts, as well as the metabolites and conjugated forms having better BBB penetration and bioavailability (Ho *et al.*, 2012; Lee & Mitchell, 2012; Spencer *et al.*, 2004). In the brain, glutathione conjugation occurs after quercetin and kaempferol is exposed to astrocytes and neurons.

2.3 Alzheimer's disease

2.3.1 Introduction

As stated previously, AD is an incurable disease with pathological features affecting the brain. These pathological features appear before any clinical symptoms. The most common clinical presentation is known as the 5 A's of AD, being anomia, amnesia, aphasia, apraxia and agnosia. The speed of progression of the illness varies between patients (Braak & Braak, 1991), with death following after ten to fifteen years (Jellinger, 2006). Risk factors taken into consideration include age, arthritis, cognitive impairment, depression, diabetes, Down syndrome, educational level, family history of dementia, head injury, high low density lipoprotein (LDL) levels, hypertension and use of non-steroid anti-inflammatory medications (NSAIDs) (Irie *et al.*, 2008; McDowell *et al.*, 1994; Riddell *et al.*, 2007; Wells *et al.*, 2015). Old age has been identified as the biggest risk factor, followed by genetics - specifically the Є4 allele of apolipoprotein E (APOE Є4) - (Corder *et*

al., 1993; Reiman *et al.*, 2009), cardiovascular and lifestyle risks (Greenberg *et al.*, 1996; Hebert *et al.*, 1995).

2.3.2 Pathology and aetiology

When comparing a person with AD to a normally aging person, they transgress into a cognitive declining mental state. It remains inconclusive what the precise cause of AD is, but various hypotheses have been developed that try to explain the disease (amyloid hypothesis, cholinergic hypothesis and others), providing continuous research on the subject. The amyloid hypothesis describes the formation of toxic Aβ deposits in conjunction with tau-proteins that lead to neurodegeneration because of Aβ overproduction or insufficient clearance (Hardy & Selkoe, 2002; Mawuenyega *et al.*, 2010). The cholinergic hypothesis is centred around the loss of cholinergic innervation of the limbic and neocortical regions of the brain. It has been found that presynaptic cholinergic markers are depleted in patients with AD. The area to undergo major neurodegeneration in the basal forebrain is the nucleus basalis of Meynert, leading to the idea that treatment with cholinergic agonists improve memory, while antagonists worsen it (Bartus *et al.*, 1982; Hampel *et al.*, 2018).

The general development of AD is assumed to start in the entorhinal cortex and hippocampus of the medial temporal lobe. From there, it spreads into the temporal, parietal and frontal neocortex of the brain (Pelgrim-Korf, 2006). During the period that the disease is fully functional, atrophy of the brain is a common sign of neurofibrillary pathogenesis. A loss in gray as well as white matter of the brain can be seen (Espeseth *et al.*, 2008). Cholinergic neurons that undergo necrosis and dysfunction in the forebrain seems to be the primary effect of neurofibrillary degeneration (Zubenko *et al.*, 1989). The first area to undergo neuronal change is the medial temporal lobe, along with the locus coereleus (LC) that is connected to the medial temporal cortex. Noradrenaline (NA) release in the LC is thought to lessen the damage to the area from oxidative stress toxicity that leads to inflammation from the high blood flow to the area (Aghajanov *et al.*, 2019). The hippocampus is responsible for regulating hypothalamic functions, regulating motor control, behaviour and learning; and regulating memory functions (Pelgrim-Korf, 2006). Atrophic damage in the hippocampal area leads to the memory deficit and lesser activation that occurs in AD (Jack *et al.*, 2000; O'brien *et al.*, 2010). In comparison, during normal aging, the hippocampus does show a decrease in volume versus the above mentioned damage (Head *et al.*, 2005).

The most prominent histopathological features of AD include NFT formed from hyperphosphorylated tau protein, which are neurotoxic, and the accumulation of neuropil threads

or plaques formed by Aβ that disrupt signalling pathways between the neurons in the neocortex and limbic regions of the brain (Ball *et al.*, 1997; Shobab *et al.*, 2005). An imbalance in the clearance and production of Aβ-peptides exist, resulting in the accumulation and aggregation of Aβ (Hardy & Selkoe, 2002; Mawuenyega *et al.*, 2010). Aβ itself is made from the proteolytic cleavage of amyloid precursor protein (APP), consisting of 40 to 42 amino acids (Castellano *et al.*, 2011; Zheng & Koo, 2011). These aggregates cause injury to synapses and finally neurodegeneration and dementia as the Aβ-aggregates are toxic in its different forms, being soluble Aβ-oligomers, intraneuronal amyloids and amyloid plaques (Mawuenyega *et al.*, 2010; Putcha *et al.*, 2011). Aβ toxicity is dependent on the presence of tau-proteins which are microtubule-associated (Roberson *et al.*, 2007). When these become hyperphosphorylated, they aggregate and form NFT-deposits in the different regions of the brain. Tau proteins can already be seen in the LC during early adulthood, but does not always mean that AD is going to be evident (Braak *et al.*, 2011; Goedert *et al.*, 2006). Formation of Aβ deposits are thought to occur before NFT's start to form, which have shown to be responsible for the destruction of nerve cells in the cortical and subcortical nuclei of the brain (Braak & Braak, 1991).

As a consequence, neurotransmitter deficits occur because of the neuronal cell loss. It is believed that the primary cause of cholinergic death and dysfunction in the forebrain is the cause of neurofibrillary degeneration (Putcha *et al.*, 2011). The further the patient has progressed in pathology, the greater the cholinergic loss is. Of these, acetylcholine receptor (AChR) and ACh loss in the brain cause severe cognitive decline (Wells *et al.*, 2015; Whitehouse *et al.*, 1982). In the raphe nuclei and LC (Ball *et al.*, 1997), serotonergic neurons and noradrenergic cells are lost, an increase in monoamine oxidase B (MAO-B) activity can be seen, glutamate and limbic pathways function abnormally, and the excitatory neurotransmitter glutamate, becomes neurotoxic as it causes an influx of intracellular calcium ion (Ca^{2+}) via NMDA receptors leading to the activation of nitric oxide (NO) synthase which generates ROS (Akaike *et al.*, 2010; Koh & Choi, 1991; Tamura *et al.*, 1992; Wells *et al.*, 2015).

2.3.3 Genetics influencing susceptibility and pathogenesis of Alzheimer's disease

Certain genotypes enhance an individual's risk for having AD. It has to be taken into consideration that genetics influence early onset AD a lot more than it does late onset (sporadic) AD (Lindsay *et al.*, 2002). If the patient has early onset AD (before age 65), it is usually caused by an alteration on chromosome 1, 14 or 21. Whereas familial Alzheimer's disease (FAD) influences both early onset AD and sporadic AD carrying it over as a dominant autosomal trait (Castellano *et al.*, 2011). Presenelin-1 (PS1) allele, found on chromosome 14, and presenelin-2 (PS2) allele found on

chromosome 1, promotes generation of $A\beta_{1-42}$ in familial AD (St George-Hyslop *et al.*, 1992; Tomita *et al.*, 1997). On chromosome 21, APP's undergo mutations responsible for FAD (Duyckaerts *et al.*, 2008). PS1 and PS2 are essential components in the γ-secretase complex that is responsible for the cleavage and release of Aβ. APOE isoforms (APOE 2, APOE 3 and APOE 4) are polymorphic proteins generated by their own three specific genotype alleles (Є2, Є3 and Є4) in the general population. These isoforms regulate cholesterol levels which modulate ß- and γ-secretase activity and production of Aβ (Castellano *et al.*, 2011; Corder *et al.*, 1993; Osenkowski *et al.*, 2008; Reiman *et al.*, 2009; Utermann *et al.*, 1980; Zannis *et al.*, 1981). Sporadic AD genetic alteration is with the APOE allele (Wells *et al.*, 2015), with the risk for having AD increasing in the order of Є4>Є3>Є2 isoforms (Castellano *et al.*, 2011; Reiman *et al.*, 2009). There exists a homozygous form of the allele, being APOE Є2/2, Є3/3 and Є4/4, as well as a heterozygous form, being APOE Є2/3, Є3/4 and Є2/4 (Huang, 2010). If the person has the APOE Є3/4 or Є4/4 gene, the chance of inheriting AD is heightened (Corder *et al.*, 1993; Jack *et al.*, 2000).

APOE genotypes strongly affect Aβ deposition, forming senile plaques and causing cerebral amyloid angiopathy (CAA) (Biffi *et al.*, 2010; Greenberg *et al.*, 1996; Shobab *et al.*, 2005). CAA is the pathogenesis describing how amyloid is spread and deposited in the brains cerebrovascular system's walls (Vinters, 1987). APOE increases a person's risk in forming AD by initiation and acceleration of Aβ accumulation, aggregation and deposition (Jiang *et al.*, 2008; Liu *et al.*, 2013). APOE 4 is not as effective as APOE 3 in Aβ clearance (Castellano *et al.*, 2011), leading to the higher toxicity levels and cholinergic loss seen in AD as ageing progresses (Espeseth *et al.*, 2008).

Peripherally APOE is generated in the liver and macrophages, responsible for mediating cholesterol metabolism (LaDu *et al.*, 1994). In the brain, the central nervous system (CNS) produces its own cholesterol (Shobab *et al.*, 2005). A synergistic association between APOE 4 and hyperlipidaemia and hypercholesterolemia that causes stroke, atherosclerosis and coronary heart disease has been found (Lahoz *et al.*, 2001; Mahley, 1988; Riddell *et al.*, 2007). This synergistic increased risk is also found in peripheral vascular disease and type 2 diabetes (Haan *et al.*, 1999). Diabetic patients with the APOE Є4 allele show a higher number of neuritic plaques (promoted by a diabetes-related factor of hyperinsulinemia, hyperglycaemia and insulin resistance), neurofibrillary tangles and CAA, than patients without the carrier gene (Greenberg *et al.*, 1996; Peila *et al.*, 2002). Lower cerebral glucose metabolism has also been seen in carriers of the APOE Є4 allele, causing oxidative stress (Irie *et al.*, 2008).

APOE is formed by astrocytes and microglia in the CNS (Aoki *et al.*, 2003; Jiang *et al.*, 2008). This production can be induced by neurological stresses or trauma for repair or remodelling.

Cholesterol is transported by APOE to neurons via APOE-receptors, a member of the low density lipoprotein receptor (LDLR) family, essential for axonal growth, synaptic formation and remodelling as it helps with learning, memory formation and neuronal repair (Mauch *et al.*, 2001; Shobab *et al.*, 2005). The APOE Є4 carrier is less effective in carrying cholesterol to the neurons for myelin replacement and repair than the Є3 isoform (LaDu *et al.*, 1994; Liu *et al.*, 2013; Mahley, 1988). This shows a lower affinity for Aβ by APOE 4, than APOE 3, suggesting a lower rate of Aβ clearance (Castellano *et al.*, 2011; Jiang *et al.*, 2008). Aβ removal could be modulated by APOE as it carries Aβ over the BBB into the systemic circulation, away from the brain, thus showing that with a lower affinity, the APOE 4 carrier impedes Aβ clearance (Beffert *et al.*, 1999; LaDu *et al.*, 1994; Mawuenyega *et al.*, 2010). When an APOE 4 fragment becomes truncated (APOE released by neurons, not astrocytes) (LaDu *et al.*, 1994), tau protein hyperphosphorylation, cytoskeletal disruption and mitochondrial dysfunction occur (Huang, 2010; Mahley *et al.*, 2006). More of these fragments were found in the hippocampus and cortex than in the cerebellum, showing neurodegeneration relating to AD (Brecht *et al.*, 2004).

Taking this into consideration, APOE Є4, together with cerebrovascular disease in conjunction with these diseases can strengthen the cognitive decline that is found in AD (Liu *et al.*, 2013). Unrelated to Aβ, APOE 4 is responsible for triggering the inflammatory cascade in the brain leading to neurovascular dysfunction (BBB-breakdown, toxic proteins from exudate into the brain as well as length reduction of capillaries) (Bell *et al.*, 2012). Neuro-inflammation contributes to neuronal damage as the APOE 4 carrier is upregulated after ischemia or oxidative stress, causing a pro-inflammatory, or a lessened anti-inflammatory function (Aoki *et al.*, 2003; Bales *et al.*, 2000; Brecht *et al.*, 2004; Desagher *et al.*, 1997). This could lead to increased cognitive decline in the elderly due to higher blood pressure in the area causing brain damage (Bender & Raz, 2012).

2.3.4 Current treatment and hypotheses for future treatment

The amount of progression of AD is measured by the use of the mini-mental state examination, determining the stage of disease and treatment that is required. The current treatment of AD only addresses the cholinergic decline of the patient. This in itself is not an effective strategy as the rates of improvement are minimal. The focus of treatment is thus on slowing the progression of the disease, so that the patient's quality of life can be maintained for a longer period. Non-pharmacological strategies are followed to cognitively stimulate these patients. It has been found that the level of education that a person has, influences the rate of degradation that a person undergoes (Wolf *et al.*, 2019). But this stimulating effect does not have as beneficial an effect as treatment to stimulate cholinergic performance (Hampel *et al.*, 2018).

Pharmacologically, acetylcholine esterase inhibitors (AChEI) (cholinomimetics) are used to delay cholinergic loss as it inhibits the breakdown of free acetylcholine in the synapse and neurons, thus leading to cognitive enhancement (Sabbagh *et al.*, 2006). It has been seen that experimentally induced Aβ deposits in the brain can be prevented by AChEI, which shows a possible reduction in disease development progression (Sabbagh *et al.*, 2006). In patients whose brains have been examined after treatment with AChEI, atrophy of grey- and white matter and metabolic changes usually seen in AD patients were reduced (Cavedo *et al.*, 2017; Krishnan *et al.*, 2003). The initial rationale behind the use of AChEI's was evaluated in a review which did not agree with all information given, warranting further research (Giacobini, 2001):

- A decrease in synthesis of ACh occurs as cholinergic neurons early on undergo selective damage.
- After reaching steady state ACh-levels with treatment of AChEI's, a short term cognitive symptomatic improvement can be seen as shown in animal and human experiments.
- As soon as therapy is discontinued, the effects of elevated ACh no longer persist, meaning the drugs are only useful as long as treatment is continued.
- Patients in early stage AD would respond to therapy with AChEI's, while late stage AD patients would not respond as effectively.
- AChEI's beneficial effect may only be limited to non-behavioural improvement of symptoms, not behavioural.

The problems with the above rationale were highlighted as firstly, in the early stage of AD, the clinical beneficial effect achieved by AChEIs is unlikely to be caused by enzymatic activity, but that there rather is a problem with sub functional activity of the cholinergic system in ACh release and storage; or an under expression of receptors in the synapse. Secondly, as there is a compensatory response to ensure sufficient hydrolysis and synthesis of ACh in the brain when enzymatic activity is unfavourable, there is an excess of cholinergic enzymes to help maintain homeostasis. This suggests that later stage AD would benefit more from AChEI-treatment than patients with mild disease (Giacobini, 2001).

Examples of AChEI's are galantamine, rivastigmine, donepezil and tacrine (Akaike *et al.*, 2010). It has also been seen that neuronal loss induced by glutamate is inhibited by AChE inhibitors (Akaike *et al.*, 2010). In a placebo study comparison with donepezil, it was found that patients with prodromal signs of AD on donepezil showed less cortical thinning and basal forebrain atrophy (Cavedo *et al.*, 2017). Patients treated with rivastigmine in clinical trials that discontinued their

treatment showed slower progression of AD than patients treated with placebo, or patients that discontinued their placebo (Sabbagh *et al.*, 2006).

Memantine, an *N*-methyl-*D*-aspartate receptor (NMDAR)-antagonist, inhibits the glutamatergic effect leading to neuronal loss (Hynd *et al.*, 2004). This drug is used in the more severe cases of AD (Wells *et al.*, 2015). It must be taken into account that these medications have a minimal effect with severe adverse effects. If the patient does not take their medication as prescribed in regular intervals, its effect is negligible.

Statins (which are lipophilic and cross the BBB) were evaluated in preclinical studies to examine their effect on distribution of cholesterol in the brain. Statins competitively inhibit the HMG-CoAR enzyme that converts HMG-CoA to mevalonate in cholesterol biosynthesis. It could thus prevent further Aβ formation. In AD clinical studies, it showed improvement, but risks and potential benefits need to be taken into account (Shobab *et al.*, 2005).

Out of the Aβ-hypothesis, the following six strategies for possible treatment have been proposed (Hardy & Selkoe, 2002):

- Partial inhibition of the γ- and ß-secretase protease enzymes to inhibit Aß formation from the cleavage of APP (Huang, 2010).
- Preventing oligomerization and enhancing Aβ-clearance from the brain. Immunisation against Aβ has been looked into, but in light of the CNS transient inflammatory response it caused, it was terminated.
- Lessening the cellular inflammatory response caused by Aβ accumulation.
- Modulating cholesterol homeostasis.
- Chelating copper- (Cu^{2+}) and zinc (Zn^{2+})-ions that Aß aggregation is dependent on.
- Preventing synaptotoxic and neurodegenerative effects triggered by Aß-accumulation. This includes use of antioxidants and use of neuroprotective compounds with neurotrophic properties.

More recently, it has been suggested by Huang (2010) that drug development focus on inhibiting cleavage of APOE, by developing an APOE-cleaving enzyme (AECE); focussing on protease inhibitors; and blockade of APOE 4 fragments interaction with cytoskeletal elements and mitochondria after truncating.

2.3.5 Reactive oxidative species in the body

All aerobic organisms need oxygen (O_2) to live. Of the total amount of O_2 in the body, less than 1% is converted to ROS. ROS include superoxide ($O_2^{\cdot}$) and hydrogen peroxide (H_2O_2). A free radical can be defined as a species that independently exists, containing a single or multiple unpaired electrons. If one of these radicals react with a non-radical, it causes the non-radical to lose an electron, becoming a radical itself (Halliwell, 1989). The human body has its own mechanisms that defend the body against ROS. ROS is used in the body during metabolism and plays a role in the regulation of immunologic defences during infection (Baynes, 2014).

For a ROS to be formed during biological redox reactions, an O_2-biradical (the normal form of oxygen in the body that has two unpaired electrons) is activated by transition metal ions in metalloenzymes like iron (Fe^{3+}) or Cu^{2+} (Fridovich, 1989). When the O_2-biradical reacts with the metalloenzyme, metal-oxo-complexes are formed that are highly reactive. The O_2 is activated for metabolism, leaving free redox-active metal ions in the body, forming ROS that causes oxidative biological damage to the biomolecules in the body (Baynes, 2014; Croft, 1998; Halliwell, 1989). Figure 2.12 shows the reduction of O_2 into ROS.

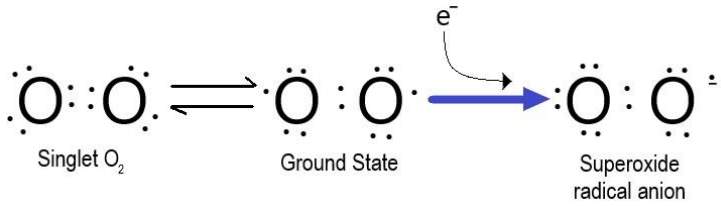


Figure 2.12: Reduction of oxygen in its ground state to superoxide radical anion (Baynes, 2014).

The $O_2^{\cdot}$ radical is further reduced to H_2O_2, which then undergoes further reduction with water as the final product (Baynes, 2014), as seen in figure 2.13:

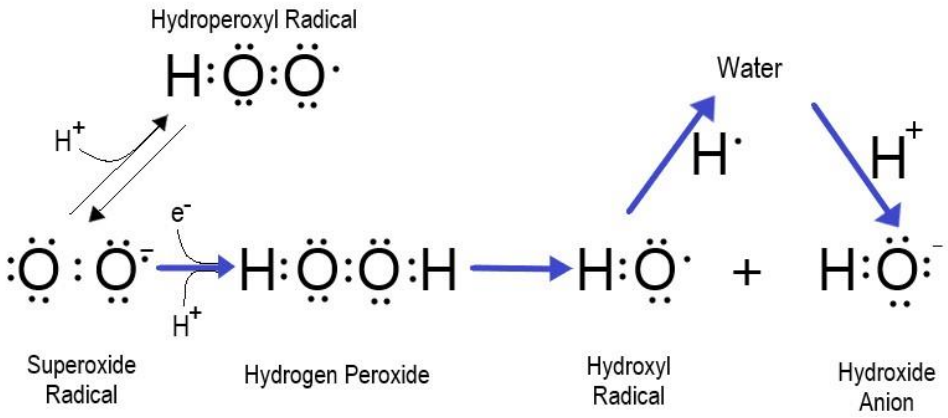


Figure 2.13: Schematic representation of the reduction of $O_2^{\cdot}$ to other radicals found in the body, such as the hydroperoxyl radical ($HOO^{\cdot}$) and the hydroxyl radical ($HO^{\cdot}$) (Baynes, 2014).

In figure 2.13, H_2O_2 is reduced by the free redox-active metal ions found in the body. This forms the $HO^{\cdot}$ radical which is the most damaging of all the radicals found in the body (Baynes, 2014).

When the antioxidant defences of the body are insufficient, oxidative stress occurs (Croft, 1998). The modern definition of oxidative stress is the imbalance that exists between oxidants and antioxidants in a living organism that favours the oxidants causing a disruption in redox signalling and biomolecular damage (Sies, 2018). The process by which damage is caused is by reaction of the $HO^{\cdot}$ with biomolecules through abstraction and addition reactions. Cell membranes consist of polyunsaturated fatty acids (PUFA) which are readily oxidised. The $HO^{\cdot}$ abstracts a hydrogen atom from the cell membrane, causing the membrane's integrity to be compromised (Halliwell, 1989). Secondary reactions take place because ion gradients cannot be upheld, leading to formation of secondary oxidation products like malondialdehyde (MDA) (Fridovich, 1989) and hydroxynonenal (HNE). Advanced lipid peroxidation end-products (ALE) are formed when MDA and HNE react with proteins, forming adducts and crosslinks. When combined with lysine residues, oxidative stress increases as has been measured in AD and the vascular wall of atherosclerotic patients. This also happens when ROS undergoes addition reactions to Phe, Tyr and other nucleic acid bases, leading to an increase in nitrotyrosine in these diseases (Baynes, 2014).

The mechanisms that are followed *in vivo* to form these radicals are the reaction of decompartmentalised metal ions with oxygen, the formation of radicals by the mitochondrial electron transport and normal enzymatic reactions (Fridovich, 1989). The following reactions are involved: A, the Fenton reaction; B, Haber-Weiss reaction and C, the metal-catalysed Haber-Weiss reaction (Aruoma *et al.*, 1989; Baynes, 2014), as shown in Figure 2.14:

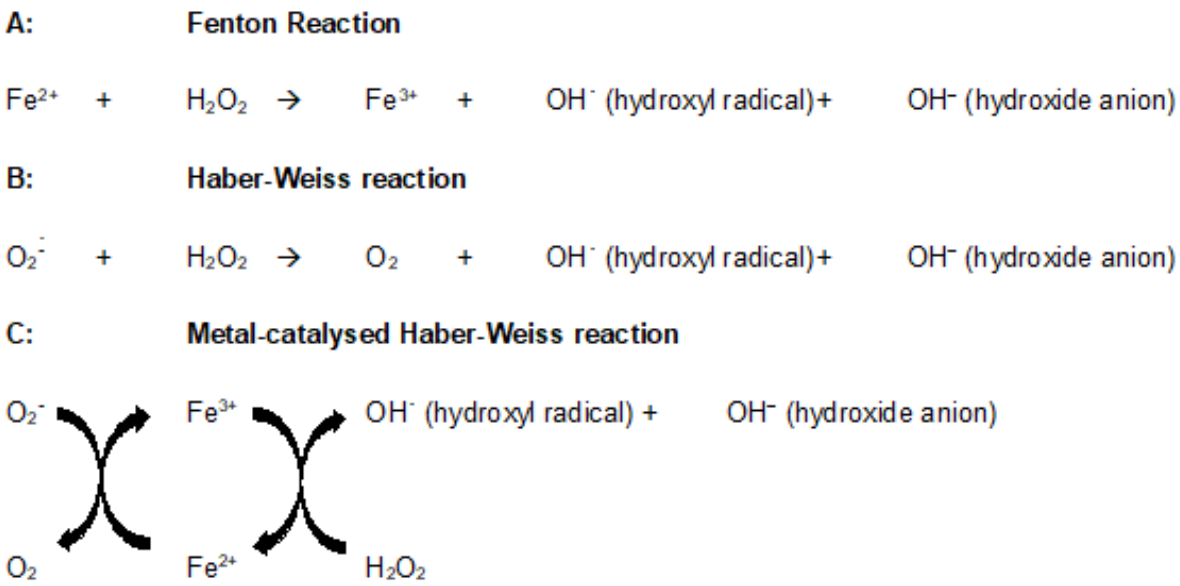


Figure 2.14: Fenton and Haber-Weiss reactions used to form ROS *in vivo* (Baynes, 2014).

These bind to metal-binding sites on proteins where the damage then occurs. $HO^{\cdot}$ that react with deoxyribonucleic acid (DNA) causes base alterations through the free radical reactive chain and strand breakage (Aruoma *et al.*, 1989; Fridovich, 1989). The body goes into an oxidative stress condition, a harmful condition. In excess, these species cause chronic damage to the tissues biomolecules (Baynes, 2014).

For this reason, the body has various antioxidant defence systems. These antioxidant defence systems are usually quite complex, but necessary (Boyer & Liu, 2004). DNA that has been damaged is repaired by excision-repair mechanisms. The first direct first-line of defence includes chelation/sequestration of redox-active metal ions, namely Cu^{2+} and Fe^{3+}, by metal-binding proteins into their inactive forms like transferrin and ferritin (Halliwell *et al.*, 1995). Heme is delivered to the liver where it is catabolised by cytochrome oxygenase (Fridovich, 1989). Albumins have Cu^{2+} binding sites that bind Cu^{2+}, removing the ions before it can be used in formation of ROS (Halliwell *et al.*, 1995). Carnosine in the brain acts as a chelator for Cu^{2+} in muscle and the brain, which could have an antioxidant effect (Baynes, 2014; Croft, 1998; Halliwell, 1989).

The second system includes enzymes that detoxify ROS and its precursors. They are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Croft, 1998; Halliwell, 1989). These enzymes can be found in the cells, mitochondria, extracellular fluid, vascular wall proteoglycans, peroxisomes, cytosol and nucleus; and the intestinal epithelial cells (Baynes, 2014).

The third line of defence includes vitamins A (retinol), C (ascorbic acid) and E (tocopherol). They work as chain-breaking antioxidants by donating a hydrogen atom to ROS that have been formed. Vitamin C is mainly used in the aqueous phase and vitamin E in the lipid phase of the body. Vitamin A is used in the retina of the eye and in the skin (Baynes, 2014; Ross & Kasum, 2002).

2.3.6 Antioxidant activity of flavonoids in Alzheimer's disease and other pathologies

AD is a neurodegenerative illness that can be worsened by oxidative stress that causes apoptotic cell death (Newman *et al.*, 2007). An increase in Aβ deposits shows an increase in oxidative stress (Smith *et al.*, 1998). It is proposed that Aβ produces hydrogen peroxide by binding to transition metals (such as Cu^{2+}), and H_2O_2 is transformed into a reactive $OH^{\cdot}$ radical (Opazo *et al.*, 2002). This causes lipid peroxidation reactions, leading to oxidative damage and oxidative toxicity in the brain (Newman *et al.*, 2007).

Plants contain different flavonoids, which makes the ingestion of different fruits and vegetables important as it influences the body's normal defence systems. Kaempferol, chrysin and quercetin (Doronicheva *et al.*, 2007) have been shown to have antioxidant activity *in vitro* and *in vivo* as radical scavengers by breaking the free radical chain reactions and chelating transition metal ions responsible for forming ROS (Croft, 1998; Halliwell *et al.*, 1995; McAnlis, 1997; Smith & Luo, 2003). They also repair damaged DNA bases and reduce single strand breakages in double DNA strings (Anderson *et al.*, 2000). Another mechanism through which these flavonoids work is by synergistically inducing the activity of endogenous antioxidants in the body such as the glutathione S-transferase enzyme (GST), which functions by increasing resistance to oxidative stress (Fiander & Schneider, 2000). For a compound to act as an antioxidant, it needs to react with different types of radicals, even when it is present in low concentrations (Croft, 1998; Halliwell *et al.*, 1995). Furthermore, antioxidants have to stabilise the radicals without further inducing oxidation activity, and the stoichiometry process needs to be balanced as the antioxidant reacts with two radicals at a time, ensuring that the second free radical formed is quenched. Flavonoids have antiradical structural elements that make them potent antioxidants. These structural elements include the presence of the *o*-diphenolic group in ring B, which is the main site of oxidative attack, and is part of the electron delocalisation process. The 2-3 conjugated double bond with 4-oxo function delocalises the electron of the aroxyl. The OH-groups in the 3- and 5-positions promotes the antiradical activity of the molecule (Lien *et al.*, 1999; Manach *et al.*, 1996; Ross & Kasum, 2002).

Flavonoids' antioxidant activity can prevent oxidative stress and thus may exert a neuroprotective effect. This is done by inhibiting the activity of enzymes that produce ROS, and inducing the antioxidant effect and expression of enzymes with antioxidant activity (Smith & Luo, 2003). A further reduction in Aβ protein through inhibitory activity could be another explanation of the neuroprotective effect of flavonoids, which may decrease the progression of the illness (Roth *et al.*, 1999). Roth *et al.* (1999) stated that kaempferol and quercetin exhibit a neuroprotective effect against Aβ induced toxicity by having fibril destabilisation and anti-amyloidogenic effects on Aβ and by inhibiting the enzyme, β-secretase (Ho *et al.*, 2012; Jiménez-Aliaga *et al.*, 2011). Cross-linking between $A\beta_{1-40}$ and $A\beta_{1-42}$ into Aβ-oligomers is prevented when quercetin concentration is present in a 1:1 ratio to the peptides (Ho *et al.*, 2012). Chrysin also exerts a neuroprotective effect because of its antioxidant activity (Kandhare *et al.*, 2014). It should be noted that if the conditions are ideal, or the concentration is high enough, the antioxidants start an auto-oxidation process, causing a pro-oxidant effect, leading to initiation of the body's antioxidant defences (Bravo, 1998; Halliwell *et al.*, 1995). Auto-oxidation is a degradation chain reaction for free radicals that undergo oxidation, especially when a double carbon bond is in the system (Loftsson, 2014). Figure 2.15 shows a general three step auto-oxidation process:

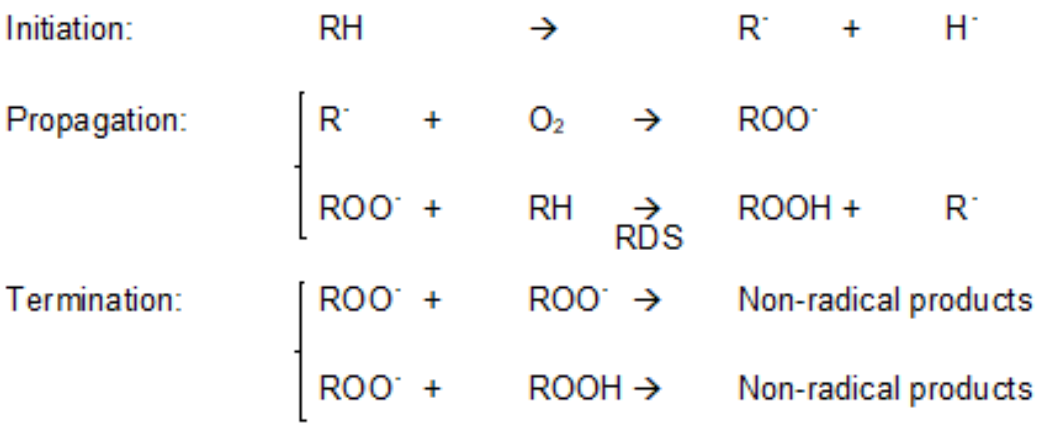


Figure 2.15: A general representation of auto-oxidation that takes place in the body (Loftsson, 2014).

In figure 2.15, during the initiation step, free radicals are formed. This happens either via thermal or photochemical cleavage of the R-H bond. Cu^{2+}, nickel (Ni^{2+}) and Fe^{3+} are catalysts at this stage. During propagation, a diatomical oxygen is added to the reaction. The second part of the propagation step is the rate determining step (RDS). Peroxyl radical (pro-oxidant) reacts with another RH, extracting the H from the RH bond, leaving another radical as product. In termination, two peroxyl radicals react to form non-radicals (Loftsson, 2014).

Figure 2.16 explains the autooxidation reaction of a flavonoid molecule:

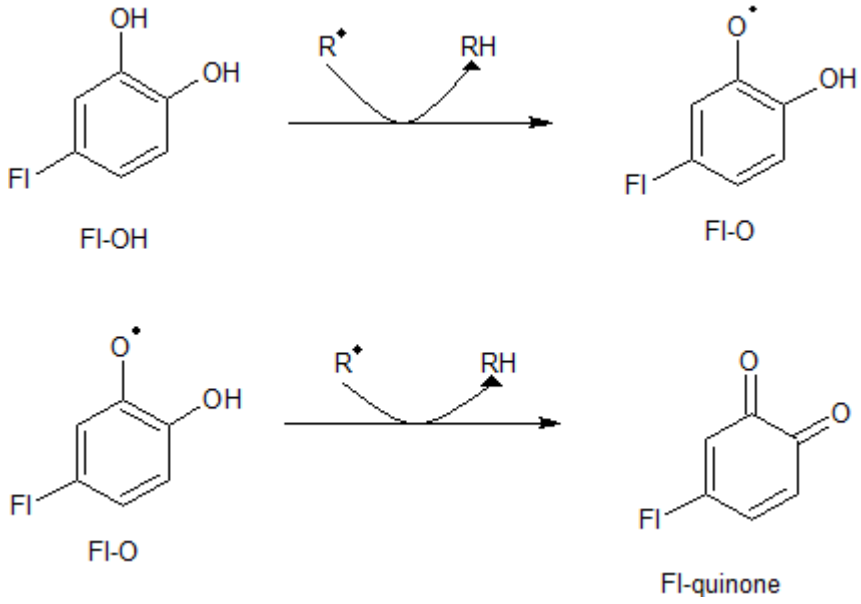


Figure 2.16: The auto-oxidation of a flavonoid molecule (Procházková *et al.*, 2011).

The flavonoid molecule reacts with a free radical, quenching the radical by giving off a Hydrogen-group. This causes formation of the flavonoid-phenoxy radical. This can cause a pro-oxidant effect. When the phenoxy-radical gives off another H, the radical effect of the free radical as well as the phenoxy-radical is quenched, leaving the flavonoid-quinone as a final product (Procházková *et al.*, 2011).

These compounds may thus play a role in managing different neurodegenerative illnesses such as AD and Parkinson's disease (Perron & Brumaghim, 2009).

Quercetin, kaempferol and chrysin aglycone have been shown to be XO inhibitors. Hypoxanthine and xanthine are oxidised to yield uric acid, causing gout. With gout, ROS production increases. This process is catalysed by the enzyme, XO, which further promotes oxidative stress (Cos *et al.*, 1998; Fridovich, 1970; Wang *et al.*, 2006). XO is converted from the xanthine oxidoreductase (XOR) enzyme. The two interconvertible forms of XOR, are XO as mentioned, and xanthine dehydrogenase (XDH). XDH is more commonly found *in vivo* in mammalian species, but is easily converted to XO when its sulfhydryl groups undergo oxidation or proteolysis (Pacher *et al.*, 2006; Zarepour *et al.*, 2010). XOR is part of the molybdenum iron-sulfur flavin hydroxylases enzymes. XOR has an effect in different areas of the body as its distribution includes the brain, heart, lungs, liver, gut, kidneys and plasma (Pacher *et al.*, 2006). Both forms of XOR can oxidise NADH, resulting in ROS formation (Zhang *et al.*, 1998). The reoxidation of XO results in the transfer of electrons to available oxygen species. Thus, for every fully oxidised XO, two H_2O_2 and one $O_2^{\cdot}$

are formed, which can cause oxidative stress (Hille & Massey, 1981; Zarepour *et al.*, 2010). This mechanism is shown in figure 2.17:

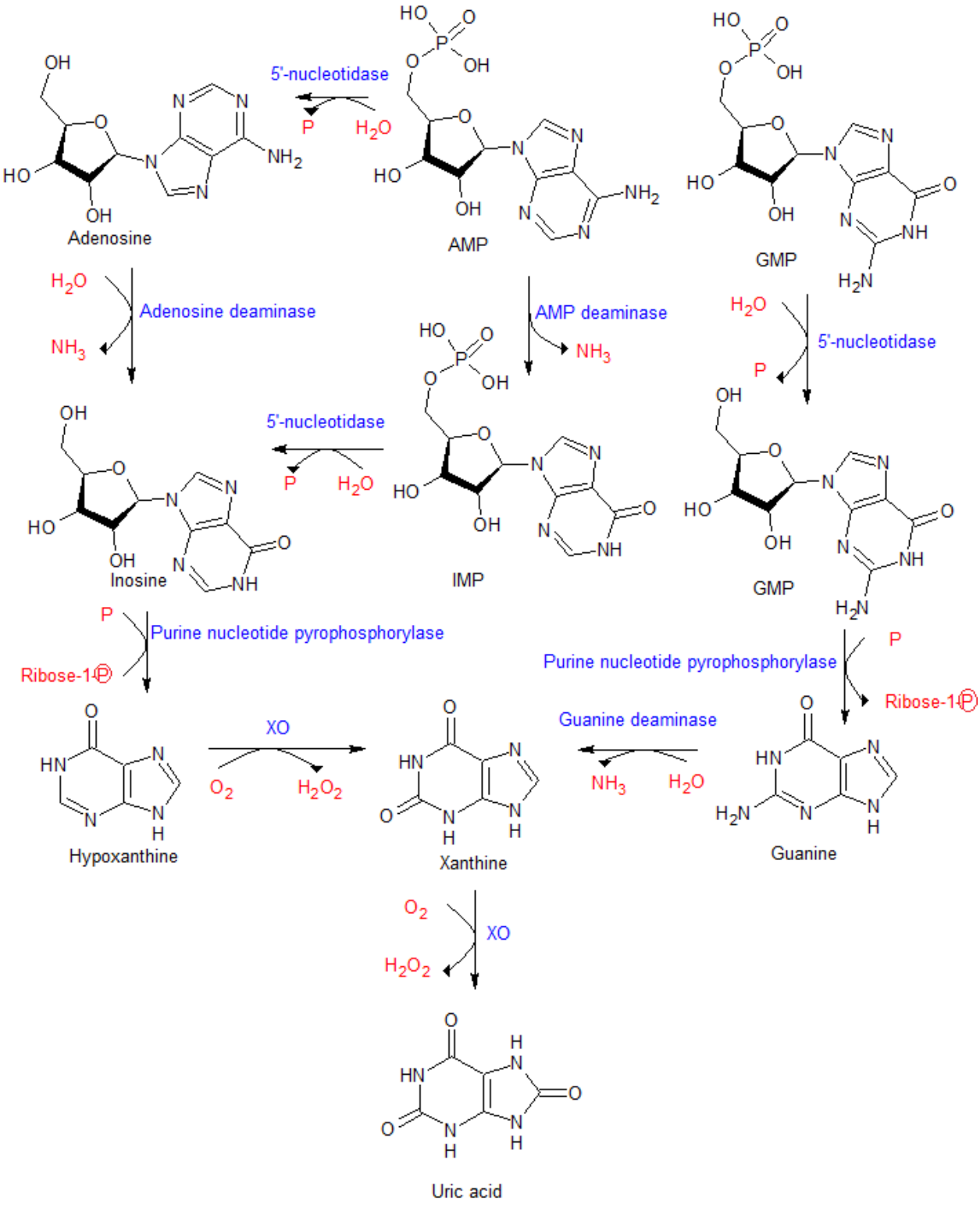


Figure 2.17: The purine degradation pathway leading to production of uric acid with byproducts of H_2O_2. Adenosine monophosphate (AMP); Inosine

monophosphate (IMP); Guanosine monophosphate (GMP) and xanthine oxidase (XO).

These radicals are part of the atherosclerosis, aging, cancer and inflammatory processes (Day *et al.*, 2000). The presence of the OH-group at the 4'-position causes the flavonol to be a xanthine oxidase inhibitor (Wang *et al.*, 2006). They also serve as antioxidants for LDL, preventing lipid peroxidation induced by Cu^{2+} (Manach *et al.*, 1995; McAnlis, 1997). If oxidation occurs, atherosclerotic plaques are formed since LDL is atherogenic (Hollman *et al.*, 1995). The mechanisms by which this protective effect is achieved are by reducing the formation of free radicals, preventing oxidation of α-tocopherol in LDL, helping to regenerate α-tocopherol and chelating the metal ions causing radical formation and oxidation. Flavonoids are also effective in inhibiting platelet adhesion, aggregation and secretion of serotonin that could worsen atherosclerosis. Kaempferol and quercetin have been shown to inhibit platelet aggregation (induced by arachidonic acid) at a concentration of 30 µmol/L (Manach *et al.*, 1996).

2.3.7 Other uses of flavonoids

Flavonoids have been shown to be protective against coronary disease and stroke (Knekt *et al.*, 1996). It is thought that the mechanism of protection could be through the antioxidative properties of flavonoids, or by inhibiting thrombogenesis, being vasoprotective and hypolipidemic (Bravo, 1998). Quercetin can be used as treatment for capillary impairment and relieving the body of insufficient blood flow through its actions produced on the veins (Erlund *et al.*, 2000).

Fiander and Schneider (2000) suggests that the progression of cancer can be decreased by alleviating oxidative stress in the body. *In vitro*, flavonoids can inhibit neoplastic cell line growth which leads to a reduction in tumour development (Manach *et al.*, 1996). Flavonoids other than kaempferol are also thought to decrease the risk of ovarian cancer. This is done by inhibiting oxidation and inflammation, altering the levels of oestrogen and other sex hormones in the body, decreasing in angiogenesis and cell proliferation as well as inducing apoptosis (Wenzel *et al.*, 2000). Flavonoids can have an effect on carcinogenesis during the initiation period by preventing activation of procarcinogens by inhibiting liver cytochrome P450 monooxygenases, detoxifying xenobiotics through activation of conjugation enzymes, increasing the rate at which carcinogens are expelled and directly interacting with carcinogens themselves (Manach *et al.*, 1996). Cancers that are affected by flavonoids include bladder-, breast-, colon-, oesophageal-, larynx-, lung-, oral- and pancreatic cancer (Ross & Kasum, 2002).

Flavonoids act synergistically in combination with vitamin C, which is a potent antioxidant in the human body. Vitamin C can possibly prevent the oxidation of the flavonoid, which then further scavenges ROS (Hertog *et al.*, 1992; Manach *et al.*, 1996).

Other biological activities of flavonoids in the body include being antibiotic, antidiarrheal, antiallergic, anti-inflammatory and antiulcerative (Bravo, 1998). Flavonoids show a reduction in COX-2 synthesis as it inhibits its production, showing its anti-inflammatory activity (Wenzel *et al.*, 2000).

CHAPTER 3 THIN LAYER CHROMATOGRAPHY

3.1 General background

In pharmaceutical analysis, chromatography is considered the most important method in use. Of the different types of chromatography, thin layer chromatography (TLC) is a high-throughput methodology that is used to deliver a bioprofile of crude extracts or components thereof (Móricz *et al.*, 2018).

Similar to all other chromatography techniques, TLC has two phases, the mobile phase (MP) and stationary phase (SP). These two phases differ from each other as the MP acts as a carrier fluid for the sample being analysed and the SP as a retention component, causing the different components of the sample to partition between these two phases through chemical binding interactions. TLC is a chromatography technique that has been developed where a thin layer (the SP) is dispersed over the surface of a plate made of glass or aluminium. TLC is generally used as a development tool as it helps with differentiation by screening for the specific compounds that are being studied and identification of active ingredients and contaminants. Compared to other chromatography techniques, it is simple, fast, easy and the least expensive for qualitative and quantitative analysis (Kaale *et al.*, 2011; Kenyon *et al.*, 1995; Kenyon *et al.*, 1999). All analysis is done visually (Hansen *et al.*, 2011).,

The SP in TLC is the silica gel, silica-gel based material (more commonly), aluminium oxide and cellulose (for polar analytes) surface area of the TLC plate. The surface area usually has a thickness of 0,25 mm consisting of particles with a size of 5-12 µm. The silica surface area (a partially dried colloidal polymerised silicic acid) can be modified upon need for separation, adjusting its hydrophilicity or hydrophobicity. It is a common occurrence that the silica is bonded to octyl or octadecyl hydrocarbon chains to be used when the MP is relatively polar (Hansen *et al.*, 2012a). Silica is a porous substance, allowing for the different substances to adsorb onto its silanol groups (-Si-OH). When the MP migrates through the SP, the entire porous area is filled allowing for contact of the analyte with the SP and MP, letting chemical bonds form according to affinity between these phases. The silanol groups allow the surface to be polar, and is a weak acid. If adjacent silanol groups have formed a hydrogen bond, the individual group itself is not as accessible for adsorption. It is because of this polarity that a more polar MP is required for normal phase chromatography (NPC). The polar interactions for NPC are dispersion interactions, dipole-dipole interactions, hydrogen bonding interactions and ionic interactions (Hansen *et al.*, 2012b).

The MP consisting of volatile substances moves through the SP through capillary forces. The MP serves the purpose of keeping analytes in solution, transporting analytes through the SP bed, contributing to separation and competing with analytes for adsorption sites. The pH is not as important as in the case of HPLC as the plates are used only once. Properties of the analyte and composition and strength of the MP solvents determine retention of the different analyte components. The more polar groups the analyte has, or the stronger the polar groups are, the more retention these groups undergo as they have a higher affinity for the SP, spending a longer time bound to the silanol groups. To achieve retention factor (R_f)-values in the optimum range, the composition and strength of the MP can be adjusted. If the solvent strength is low, the R_f-value is smaller, and the reverse is also true. If there is no specified MP, the USP suggests starting with a chloroform 180 v/v: methanol 15 v/v: water 1v/v mixture (USP, 2012; USP, 2019). If tailing is evident, there is usually a strong ionic interaction between the acid silanol groups of the SP and amino groups of the analyte, resulting in a greater degree of retention. To lessen this effect, it is advised to add a basic component (ammonia or a volatile amine like diethylamine) that competes with the amino group in binding with the silanol group. Another method is adding a volatile acid like formic acid (FA) or acetic acid (AA) that decreases ionic interactions and suppressing ionisation of silanol groups. Carboxylic groups also show a strong retention factor. This is countered by the addition of a volatile acid (Hansen *et al.*, 2012a).

NPC is the more commonly used separation technique in TLC. For this type of chromatography, the SP is more polar (for example unmodified silica gel), while the MP is more non-polar, consisting of organic solvents (for example heptane-ethyl acetate or heptane-propanol mixtures). The polar SP will thus retain analytical components that have polar groups, while non-polar analytes (that has less affinity) will migrate more speedily through the SP, at the same speed (or a bit slower) than that of the MP as it is not retained (Hansen *et al.*, 2012a).

In comparison to NPC, RPC is becoming more popular and differs from NPC as it uses more aqueous MPs (water mixed with methanol [Me] or acetonitrile [ACN]) and a modified SP (for example silanised silica gel) (Hansen *et al.*, 2012a). During reverse phase-TLC (RP-TLC) the hydrophobic or apolar analyte groups undergo stronger retention than their polar counterparts. This ensures the need for a water miscible organic solvent such as Me, ACN or acetone. R_f-values are larger when the ratio of organic solvent increased, compared to its water counterpart. Retention adjustments are achieved by the addition of acidic solvents or ammonia and ammonium acetate salts (Hansen *et al.*, 2012a; Kenyon *et al.*, 2001).

Different visualisation techniques are used as the separate components cannot always be seen without assistance, depending on the individual properties of the samples being tested (Kenyon *et al.*, 1995). If the components are coloured, it can immediately be seen. If it is known that the

sample being tested is not visible after separation, a phosphor group is added to the silica of the SP during manufacturing. If the substances absorb UV-light or are fluorescent, exposing the TLC plate to germicidal light for fluorescence under a UV-C-lamp of 254 nm, or a UV-A-lamp with 354 nm wavelength will visualise the components (Kenyon *et al.*, 1995; Kenyon *et al.*, 2001). Another approach is spraying the plate surface with different reagents that form derivatised complexes with the components, visualising it immediately, or after exposure to UV-light afterwards (Aranda & Morlock, 2006; Hansen *et al.*, 2012a). Examples are iodine and iron (Kenyon *et al.*, 1995).

R_f-value is the retention factor used to describe the retention that occurred. It can be better defined as the movement of sample zone divided by movement of the developing solvent (Kenyon *et al.*, 1999). Equation 3.1 describes R_f:

$$Rf = \frac{a}{b} \tag{3.1}$$

Equation 3.1: Formula for calculating the retention factors of the different components of a sample after TLC (Hansen *et al*., 2012a).

In equation 3.1, R_f represents the retention factor; *a* is the length between the baseline and centre of a component spot after separation and *b* is the distance between the baseline and the MP front line after development (Hansen *et al.*, 2012a).

All values calculated are between the ranges of 0 to 1, with the ideal range between 0.2 and 0.8. If the development chamber (DC) is saturated and development of the TLC plates are repeated, the same components should have the same R_f-value. The lower the value, the more retention the component underwent, while at a higher value, migration was more in par with the MP movement with less retention occurring (Hansen *et al.*, 2012a).

3.2 Procedure

3.2.1 Development chamber

- When setting up the DC, a filter paper is placed on the inside against the wall of the DC to help with spreading of the MP in the space being used for chromatography, as well as becoming saturated with the MP as well (Hansen *et al.*, 2012a).

- Less than 1 cm of the bottom of the DC is filled with MP as to ensure that when a plate is placed inside, the sample is not washed off the surface, contaminating the MP (Hansen *et al.*, 2012a).
- The DC is covered and left to stand for a few minutes to ensure saturation of the atmosphere in the DC with the MP being used is achieved (Hansen *et al.*, 2012a).
- As soon as saturation has occurred, the DC can be used for chromatographic separations (Kenyon *et al.*, 1995).

3.2.2 TLC-plate

- Using a pencil, a line is drawn on the surface of the TLC-plate, 1-2 cm from the bottom. All sample and other labelling is also to be done in pencil (Hansen *et al.*, 2012a; Kenyon *et al.*, 1995).
- When plotting the samples to be analysed, a tiny dot is made on the base line drawn. A micropipette is to be used (Hansen *et al.*, 2012a; Kenyon *et al.*, 2001).
- In the event of band broadening occurrence, the samples should not be spotted less than 1 cm from each other (Hansen *et al.*, 2012a).
- After spotting, evaporation of the solvent should first occur until the plate is dry. The TLC-plate can then be placed in the DC that has been set up (Hansen *et al.*, 2012a; Kenyon *et al.*, 1995; Kenyon *et al.*, 2001).
- When placing the plate in the DC, the MP should not be above the pencil line. After placing the plate vertically in the DC, cover the top again to ensure that the DC stays saturated and the MP does not evaporate from the plate during development (Hansen *et al.*, 2012a).
- Separation starts as the MP slowly moves upwards through the SP. The compounds will move at different rates with the MP, according to their affinities for the MP.
- As soon as the MP has moved two thirds or three quarters of the plate length, the plate is removed and the position of the MP front is marked with pencil. The TLC plate is given time so the MP can evaporate from its surface (Hansen *et al.*, 2012a; Kenyon *et al.*, 1995).
- The properties of the compounds determine what visualisation techniques will be appropriate for viewing (Kenyon *et al.*, 1995). In this instance, visualisation was obtained by exposing the developed TLC-plates to iodine and/or visualising it under UV-light (Kenyon *et al.*, 2001).

- After marking the end spots, the R_f-value can be determined to identify the compounds in a mixture, which is specific to the environmental conditions under which the separation has been done.

Figure 3.1 and 3.2 are graphical representations of the setup, development and analysis of TLC plates:

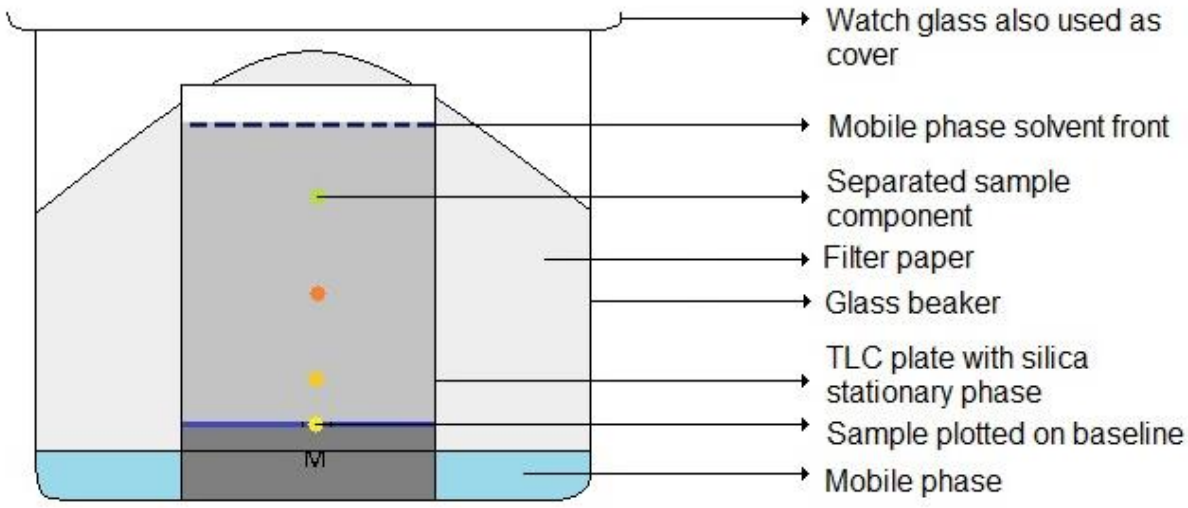


Figure 3.1: Setup for normal thin layer chromatography, including the development chamber and TLC-plate as adjusted from Du Plessis (2018).

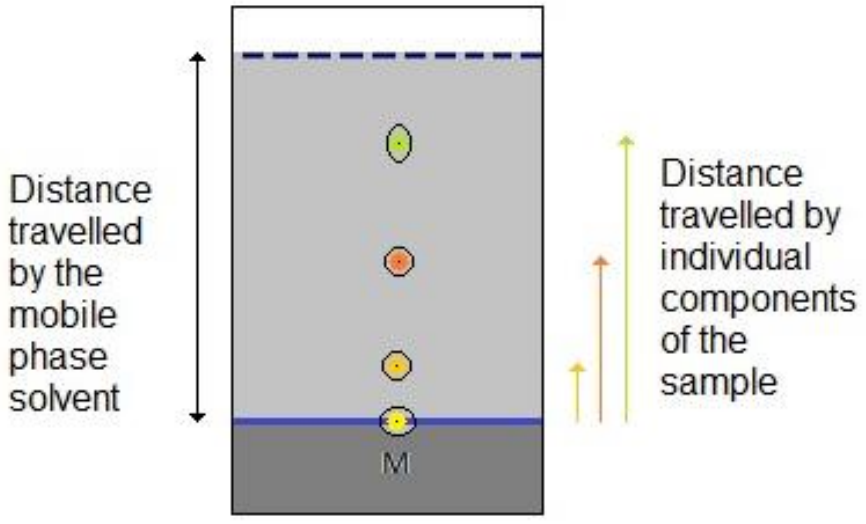


Figure 3.2: Important parameters for calculating the retention factor of different components shown on a TLC-plate (Du Plessis, 2018; Hansen et al., 2012a).

3.3 Chemicals and materials

For development of a TLC-method the following chemicals, materials and standards as shown in table 3.1 were used:

Table 3.1: Chemicals and materials for TLC-method development.

Chemicals and materials for TLC-method development:			
Item:	**Grade:**	**Batch number:**	**Company:**
Materials:			
TLC Silica gel 60 F_{254} plates	Aluminium backing	HX57994151	Merck©
Apparatus:			
UV-light	254 nm 354 nm		
Chemicals:			
Chloroform ($CHCl_3$)	Gold Line	C05201102500	ACE© (Associated Chemical Enterprises)
Methanol (CH_3OH)	Gradient grade for liquid chromatography	I0930107 803	Merck©
Ethyl Acetate ($CH_3COOC_2H_5$)		191115EA	Rochelle Chemicals©
Toluene ($C_6H_5CH_3$)		SAAR6081040LC	Merck©
Formic acid 85% (CH_2O_2)	Gold Line	F0950CC02500	ACE©
Ethanol 95% (C_2H_5OH)	Industrial	8972	ACE©
Acetic Acid glacial (CH_3COOH)	Platinum line	A0011FC02500	ACE©
Resublimed Iodine (I)		27442	SAARCHEM©
Standards:			

Chrysin or 5,7 - Dihydroxyflavone ($C_{15}H_{10}O_4$)	97%	C80105-25G	Sigma-Aldrich©
Quercetin or 2-(3,4-Dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one,3,3',4',5,6-Pentahydroxyflavone, Quercetin-3-O-rhamnoside ($C_{15}H_{10}O_7$)	HPLC (>95%)	Q4951-10G	Sigma-Aldrich©
Kaempferol ($C_{15}H_{10}O_6$)	HPLC (>90%)	K0133-10MG	Sigma-Aldrich©

*Abbreviations for the chemicals used during experimentation were toluene (T); chloroform (CF); Ethyl acetate (EA); Acetic acid (AA); Methanol (Me); Ethanol (E); Formic acid (FA) and Dichloromethane (DCM). Standards used during experimentations were abbreviated to chrysin (C); Kaempferol (K) Quercetin (Q) and standard mixture (M).

3.4 Method development

From previous literature (Móricz *et al.*, 2018; Panchal *et al.*, 2017; Williams *et al.*, 1997), different mobile phases were set up to use for chromatographic separations from the different chemical solvents as listed in table 3.1. The following list of MP ratios were used and adjusted on a trial-and-error basis after evaluating the success of the different compositions to effectively separate the compounds:

A: CF (75): EA (10): Me (15)

B: T (80): AA (20)

C: T (60): EA (30): FA (10)

Before testing, individual standards (small unspecified amount) were dissolved in Me (5 ml) (gradient grade for HPLC) and one standard mixture (mixed from the individual dissolved standards) was prepared. After having tested each of these mobile phases, it was decided to test each individual solvent as a MP, with the time of elution of the standards with the individual chemical MPs listed in the order of quickest to slowest:

1. EA
2. C
3. T
4. Me
5. AA
6. FA

This helped to discern the performance of individual MPs and gave an indication of how the MPs should be combined in the next step. The following 50:50 ratio MPs were used with the second component always being EA:

- FA (50): EA (50)
- EA (50): EA (50)
- Me (50): EA (50)
- T (50): EA (50)
- CF (50): EA (50)

Out of the previous two sets of TLC-data, it was decided to use the following MPs:

- T (80): AA (20)
- T (60): EA (30): FA (10)
- T (50): E (50)
- CF (50): EA (50)
- CF (40): E (40): DCM (20)

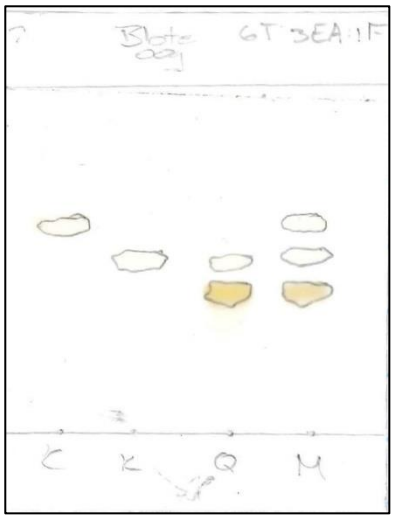
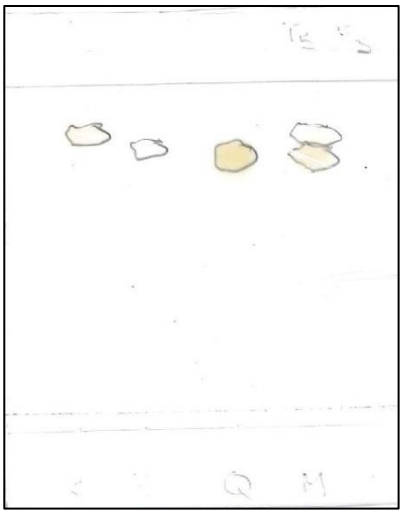
A final T:E ratio of T (90): E (10) was mixed and run to establish what this final MP adjustment effect will have on the separation of the standard components being separated.

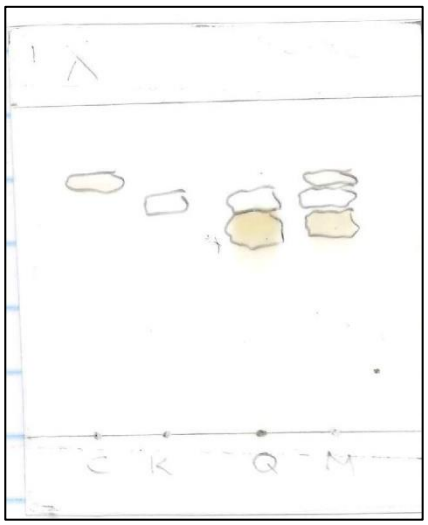
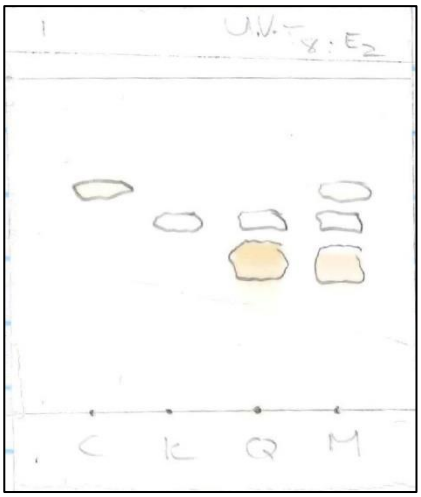
3.5 Results

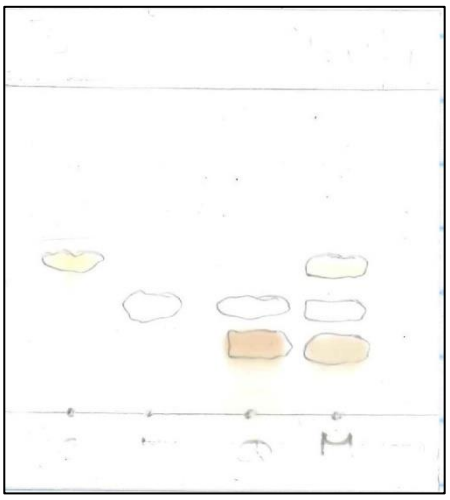
All TLC-development plates for stage 1, 2 and stage 3 are available, but are not included in this dissertation. Stage 3 plates that were unsuccessful include T80: AA20; CF40: E40: DCM 20 and CF50: EA 50 MPs.

To visualise all components on the TLC plates after separation and drying, the plates were exposed to iodine in an iodine chamber as well as visualised under a UV- and germicidal-light with best results delivered by the UV-light (254 nm). Results are given in table 3.2:

Table 3.2: Results of NPC for TLC development.

Plate:	**Specifications which include samples, solvent and mobile phase:**
	Samples: 1. C 2. K 3. Q 4. M Solvent: • Me MP: • T60 • EA30 • FA10
The MP T60: EA30: FA10 gave efficient separation and is discussed in the conclusion.	
	Samples: 1. C 2. K 3. Q 4. M Solvent: • Me MP: • T50 • E50

<table>
<tr><td> 
</td><td>Samples:
1. C
2. K
3. Q
4. M
Solvent:
• Me
MP:
• T70
• E30</td></tr>
<tr><td colspan="2">The T70: E30 MP showed limitef separation of C, K and Q as well as all three standards present in the M. Further adjustment was necessary.</td></tr>
<tr><td> 
</td><td>Samples:
1. C
2. K
3. Q
4. M
Solvent:
• Me
MP:
• T80
• E20</td></tr>
<tr><td colspan="2">This separation proved efficient, especially with the standards separated in the middle area of the plate. To see if further separation is possible, it was decided on adjusting the MP to T90: E10.</td></tr>
</table>

<table>
<tr>
<td>  </td>
<td>Samples:
1. C
2. K
3. Q
4. M
Solvent:
• Me
MP:
• T90
• E10</td>
</tr>
<tr>
<td colspan="2">The above separation was efficient, but caused more tailing and band broadening, making it less effective than its T80: E20 counterpart.</td>
</tr>
</table>

3.6 Discussion

As seen above, two methods were effective in separation of the three flavonoid compounds. These are the T80: E20 and T60: EA30: FA 10 MPs. Both MPs could thus be used depending on the solvents available and the researcher's discretion of effectiveness. Visualisation was possible without any aids, but the separated components were very light, especially C, leading to the use of iodine exposure and visualisation under a UV-light (254 nm). Below the different plates and their usefulness are discussed, starting with figure 3.3, which is the T90: E10 MP:

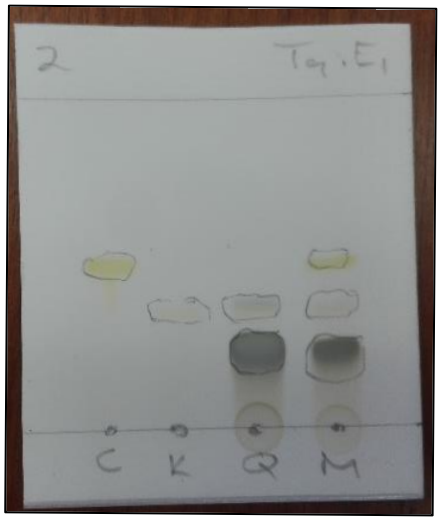


Figure 3.3 TLC plate separation after exposure to iodine (5-30 s) for visualisation using the mobile phase toluene 90: ethanol 10.

The separation achieved using T90: E10 MP was effective, but it was decided not to use this MP as it shifted the whole separation area lower, achieving smaller R_f-values, closer to 0.2. It did show better separation than T80: E20 as the components are further spaced from each other and could thus be clearly seen. The plate in figure 3.4 proved more useful:

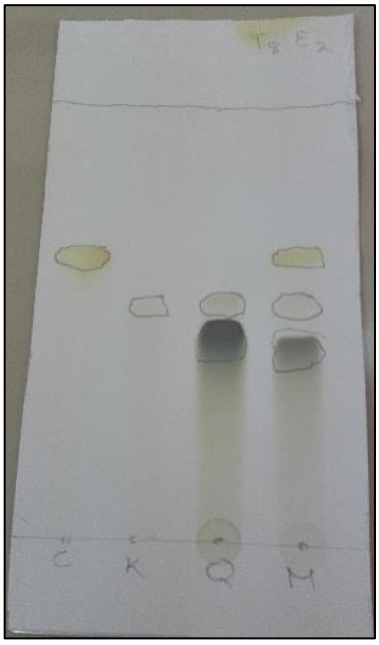


Figure 3.4: TLC plate separation after exposure to iodine (5-30 s) for visualisation using the mobile phase toluene 80: ethanol 20.

As can be seen, all three components of the M (C, K and Q) are sufficiently separated with the components closer to the middle area, also being clearly visible. This shows that the adjustment between the T: E ratio causes an increase in the R_f-value when the E is increased and T is decreased. When comparing this result to the T70: E30 MP as seen in the results, the T70: E30 did not achieve sufficient separation, showing that the most effective range for the ratios should be T80-90: E20-10. In these plates, the C shows as a yellow spot, the K as a very light grey spot and the Q shows as a bigger and wider dark grey spot.

In figure 3.5 MP T60: EA30: FA 20 is discussed:

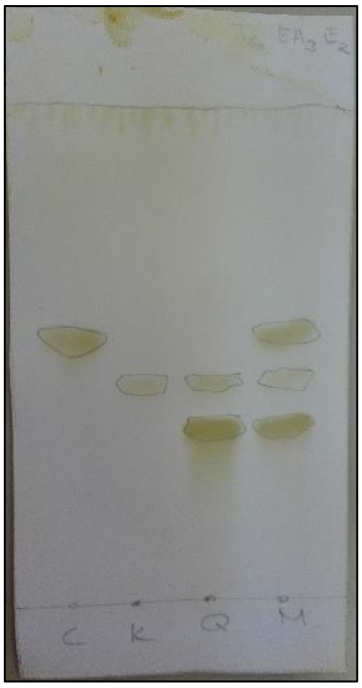


Figure 3.5: TLC plate separation after iodine exposure (5-30 s) for visualisation using the mobile phase toluene 60: ethyl acetate 30: formic acid 20.

After separation, visualisation without any assistance was quite difficult as the spots were not brightly coloured. It was necessary to expose the plate to iodine in an iodine chamber to achieve the above visualisation (figure 3.6). No adjustments were further required for the MP.

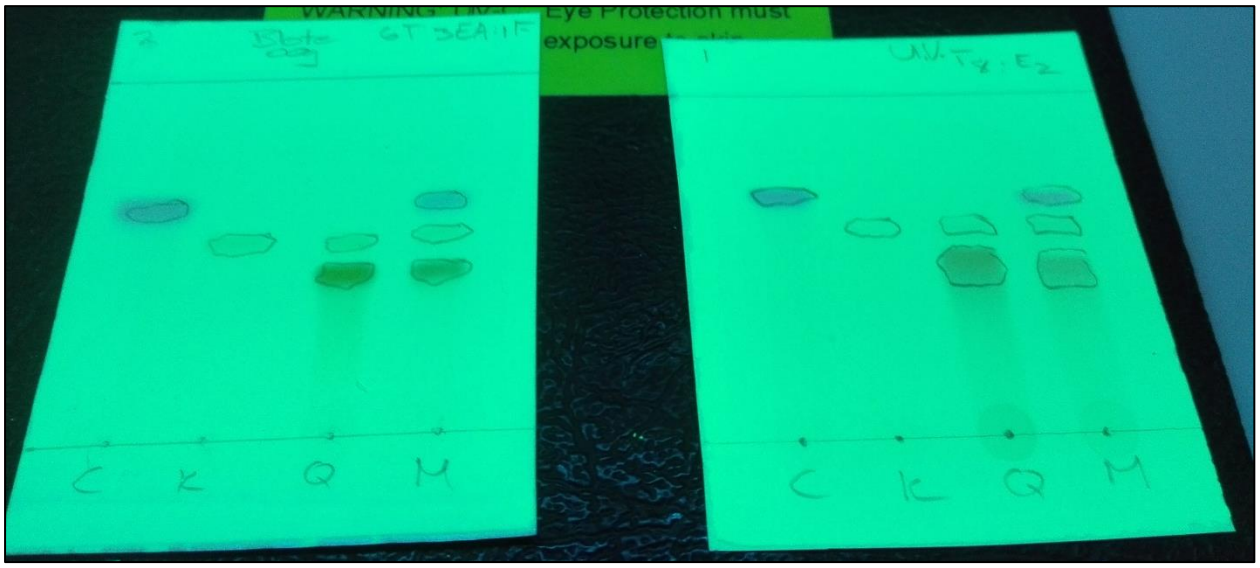


Figure 3.6: Visualisation using 254 nm UV-light for TLC plates using two different mobile phases.

Under the UV-lamp, in the T60: EA 30: FA 10 MP plate, the C is shown as a blueish-purple dot, K is a very light yellow colour and Q shows as a darker yellow colour. Compared to plate one, plate two also showed C as a blueish-purple dot, K as a very light dot and Q as a wider and longer greyish dot.

From the results, it was seen that the Q standard contains traces of K and is later discussed as part of the HPLC results.

CHAPTER 4 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND METHOD VALIDATION

4.1 General background

HPLC (high performance liquid chromatography), also known as liquid chromatography (LC), is the most common analytical method used in pharmaceutical chemistry to date. Like all other chromatography techniques, it is based upon the use of a mobile phase (MP) in conjunction with a stationary phase (SP) that separates the components of the sample being screened. When this method was used for the first time, it was known as high pressure liquid chromatography, reflecting the high pressures used in this system. After focus was shifted to the quality of the separations, the name was changed to HPLC. When very high pressures are used, it is known as ultrahigh performance liquid chromatography (UHPLC) (Hansen *et al.*, 2012d).

For HPLC, the mobile phase used is forced through the stationary phase (in this case being either a normal phase [NP] or reverse phase [RP] packed column) filled with a material that retards the pressurised progression of the analytes which have been injected into the column. The column itself is usually manufactured from steel in the range of 5-25 cm, the SP is found packed into the inside. As the MP enters the column, the analyte is injected at the front part of the column used. Inside the column, the analytes are separated into its different components by the SP for analysis after screening. After reaching the outlet of the column, the eluted components are detected by the detector to which it is connected. All components of the column are specifically chosen and setup to the specifications of the experiment that is being done. The three main parts of HPLC can be seen as the solvent delivery system, the chromatograph separation column, and the detector system (Hansen *et al.*, 2012d).

Physicochemical properties of the solvent used as MP that play a role in separation are solvent viscosity (η), the dielectric constant (Ɛ), and superficial tension (γ) of the system. This is important when selecting the MP and the solvent for the analyte as the analyte solvent should be miscible with, and not show stronger elution than the MP. General requirements to be taken into consideration during the selection process of the MP, are (Hansen *et al.*, 2012d):

- That no detector response should be obtained from the selected solvent.
- That all solvents used should have a degree of purity deemed satisfactory. These are usually classified as HPLC grade.
- That low viscosity is a general requirement for the solvent in use to ensure that back pressure during analysis is minimised.

- Solvents are to be as environmentally friendly as possible, including having low toxicity, being inflammable and nonreactive, as well as easily disposable after use.

The volume of injection is in the range of 5-100 µL and is injected into the MP before the column inlet under high pressure. As the analyte passes through the column, the electronic response supplied by the detectors is in the form of a chromatogram (Hansen *et al.*, 2012d).

In the case of RP-HPLC, the MP could contain an aqueous buffer solution. A buffer salt is dissolved in HPLC grade water beforehand. This is then mixed with an organic modifier till the desired solvent strength is reached. If there is a chance for precipitation occurring, or small particles forming, it has to be filtered through a filter with a pore size of 0.45 µm. After this, degassing has to occur, as to prevent the dissolved air from forming air pockets during use (Hansen *et al.*, 2012d).

It is of importance to know which parameters affect the quality of separations, as without this, optimisation cannot occur. These are the flow rate, the column, the pumps, the detector, the injectors, the MP and solvents used for solvent preparation (Hansen *et al.*, 2012d). During the setup of the HPLC system, delivery of MP to the column can be adjusted. This is defined as the flow rate of the MP, ranging from 0.01-10 ml/min. When the MP is delivered to the system as a constant composition (for example a mixture of methanol 50%: HPLC water 50%), it is known as isocratic elution. (Hansen *et al.*, 2012d). When there is a constant change in composition of the MP, it is called gradient elution.

During delivery, back pressure (the resistance that opposes flow through a confined space, resulting in friction that decreases pressure supplied by the initial force) occurs because of the small particle size of the SP packing material. This back pressure ranges from 3-30 MPa, decreasing effectiveness of the separation by decreasing the flow rate. This back pressure is usually visible when the analyte has too many components (as in the case of plant samples that sometimes overload the system), or the elution time is not long enough for complete separation to be achieved (Hansen *et al.*, 2012d).

According to Hansen *et al.* (2012), the importance of HPLC in drug development and research can be defined as it:

- provides methods that are accurate, precise and robust when used with UV detection when quantitative analysis of pharmaceuticals is required;
- helps in decomposition determinations as it is more than suitable for determination of stability of pharmaceuticals and their drug substances; and

- is effectively used in determination of drugs and their metabolites when testing biological samples.

4.1.1 Published methods used for HPLC analysis

Published methods used for HPLC analysis of flavonoids, specifically chrysin, kaempferol and quercetin were reported in the following studies:

- In a study done to extract flavonoids from onions using microwave extraction, quercetin (Q) and its derivatives, kaempferol (K) and myricetin were extracted for analysis. The wavelength of detection was set to 360 nm and the column used was a Purospher Star® RP-18 end-capped column (250 mm x 4 mm; 5 µm particle size). The temperature was kept at 37 °C and the flow rate at 1 ml/min. A MP consisting of (A) acidified water (0.5% formic acid [FA]) and (B) 100% acetonitrile (ACN), as well as a gradient setup was used (Vian *et al.*, 2009).
- In a study done focussed on extracts from spearmint leaves, analysis was done on an HPLC. The column used was an Eclipse® XDR-C_{18} reversed phase column (25 cm x 4.6 mm with 5 µm particle size). The MP consisted of (A) trifluoroacetic acid 2.5 pH in deionized water and (B) 100% Me. The wavelength of detection was set to 280 nm. Temperature was kept at room temperature and the flow rate was set to 1.0 ml/min (Bimakr *et al.*, 2011).
- In a study that investigated foods infused with elderberry flowers or fruits, the column used was a Zorbax® C_{18} column (4.6 mm x 150 mm, 5 µm particle size). The MP consisted of (A) 0.5% acetic acid in water and (B) ACN. The flow rate was set to 1.5 ml/min and a gradient method was used. The wavelength of detection was set at 285 nm and 355 nm depending on the flavonoid group being analysed (Oniszczuk *et al.*, 2016).
- In a study using supercritical fluid extraction of Q from onions, the column used was a BetaBasic® C_{18} column (250 mm x 4.6 mm; 5 µm particle size). The MP consisted of a mixture (2:3 v/v) of aqueous phosphoric acid: Me. Flow rate was set to 1 ml/min and the wavelength of detection to 280 nm (Martino & Guyer, 2004).
- In another study focussing on extractions from onions, the column used was a reverse-phase Eclipse® XDN-C_{18} stainless steel column (4.6 mm x 250 mm; 5 µm particle size). The MP consisted of (A) deionised Milli-Q water adjusted to a pH of 2.5 using ortho-phosphoric acid and (B) ACN. A gradient method was employed during analysis with a flow rate of 1 ml/min and the wavelength of detection set to 370 nm (Roldán-Marín *et al.*, 2009).

- This last study focused on analysis of chrysin (C) derivatives using HPLC. The column in use was a Nova-Pak® C_{18} column (150 mm x 3.9 mm; 4 µm particle size). The MP consisted of (A) Me and (B) 10 mM phosphoric acid (H_3PO_4) (This was the MP focussed upon). The flow rate was set to 1 ml/min and the wavelength of detection to 280 nm (Kim *et al.*, 2002).

These methods were evaluated and adapted where necessary for use in this study.

For validation purposes, a linear regression analysis was done using the data of the peak area compared to the concentration. The linear calibration curve drawn was from standards ranging from 10 µg/ml to 1 000 µg/ml.

4.2 Chemicals and materials

Table 4.1 shows all the different standards, chemicals and apparatus used for the HPLC technique employed during this study:

Table 4.1: Chemicals and materials used during development of the HPLC technique.

Chemicals and materials for HPLC-method development:			
Item:	**Grade:**	**Batch number:**	**Company:**
Chemicals:			
Acetonitrile (CH_3CN)	Gradient grade for liquid chromatography	I0978330 842	Merck©
Methanol (CH_3OH)	Gradient grade for liquid chromatography	I0930107 803	Merck©
Orthophosphoric acid (H_3PO_4) 85%	Gold Line	7 100	Associated Chemical Enterprises©
HPLC H_2O from Millipore Milli-Q	HPLC grade (18Ω)		
Standards:			
Chrysin ($C_{15}H_{10}O_4$)	97%	C80105-25G	Sigma-Aldrich©
Quercetin	HPLC (>95%)	Q4951-10G	Sigma-Aldrich©

($C_{15}H_{10}O_7$)			
Kaempferol ($C_{15}H_{10}O_6$)	HPLC (>90%)	K0133-10MG	Sigma-Aldrich©

The HPLC system used was a Hitachi Chromaster chromatographic system. The system consists of a 5410 UV-detector, an autosampler (5260) with a sample temperature controller and a solvent delivery module (5160). The column used for analysis was a Kinetex® EVO C_{18} column (250 mm x 4.6 mm; 5 µm particle size and 100 Å pore size).

All standard analytes (C, K and Q) were reconstituted using the following procedure:

- 100 mg of the standard was dissolved in 100 ml Me, yielding a solution containing 1 mg/ml.
- This solution was filtered using a 0.45 µm filter.
- 1 ml of the stock solution was further diluted with 100 ml Me, yielding a solution containing 10 µg/ml.
- This enabled the use of either the 1 mg/ml solution, or the 10 µg/ml standards.
- The standard mixture of the single standards (M) was composed from the single standard solutions.

It was decided to use the MP consisting of 10 mM H_3PO_4 as in the study done on analysis of C on HPLC (Kim *et al.*, 2002). The ratios were adjusted from 10:90, 20:80, 30:70, 40:60, 50:50, 60:40, 70:30, 80:20 and 90:10. The flow rate was set to 1 ml/min and the wavelength of detection to 280 nm. The injection volume for standards was 10 µL per sample. Starting this procedure, full run times were set to run at separate run times, being repeated as 30 min, 60 min and 10 min. The MP ratios that delivered the best results were 30:70, 40:60 and 50:50. The 60 min run times were too long and the 10 min too short, with the 30 min showing sufficient separation in the allotted time. It was decided to replace the Me with ACN and running the standards for 60 min. Among these three ratios, it was decided on using the 50:50 ratio. Upon further testing, this MP was adjusted to a ratio of 10 mM H_3PO_4: Me: ACN (50:25:25). This MP provided sufficient separation in the 30 min elution time run. This showed sufficient separation in the first 20 min, but to compensate for possible unknowns, the run time was set to 30 min.

4.3 HPLC results

Sample chromatograms for each of the three individual standards (C, K and Q) are shown below (figures 4.1-4.3), as well as for the mixture (M) (figure 4.4). To identify each compound in the single standard mixture, the individual compounds' retention times were compared to the retention times (Rt) seen in that of the single standard mixture.

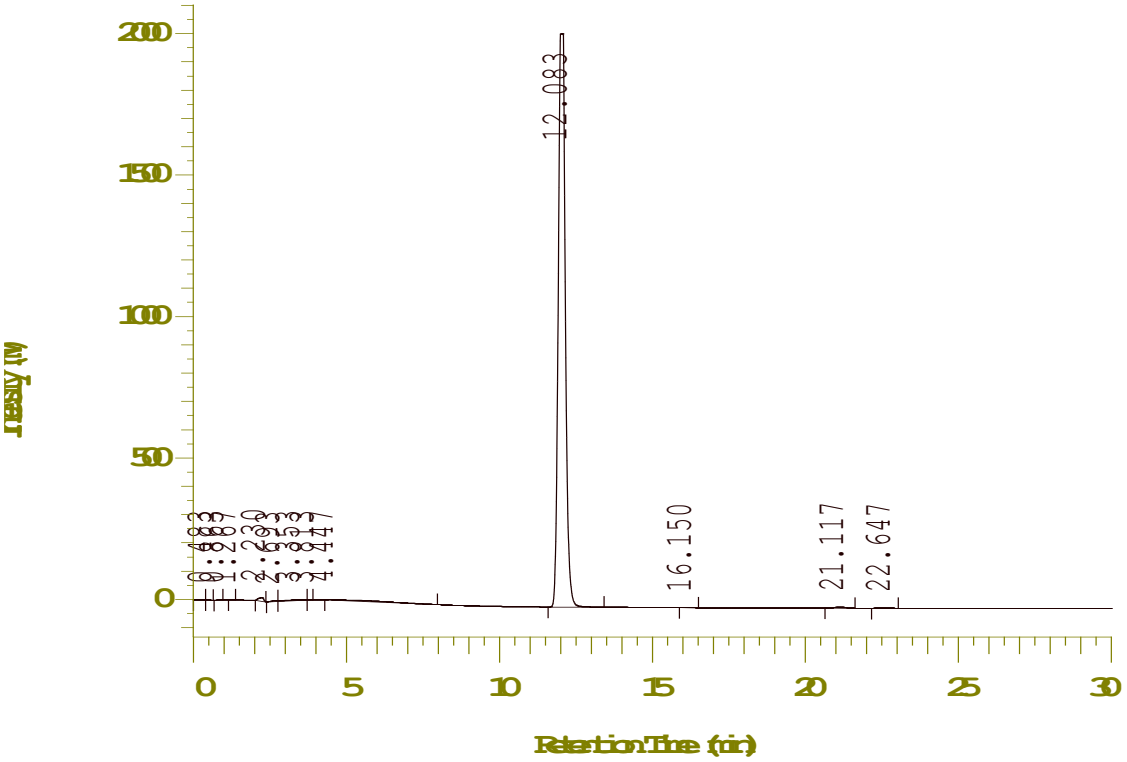


Figure 4.1: A chromatogram of a sample containing C (chrysin) with a concentration of 1 mg/ml. The MP consisted of 10 mM H_3PO_4: Me: ACN (50:25:25).

C is shown at 12.083 min. Trace amounts of other unidentified compounds can also be seen.

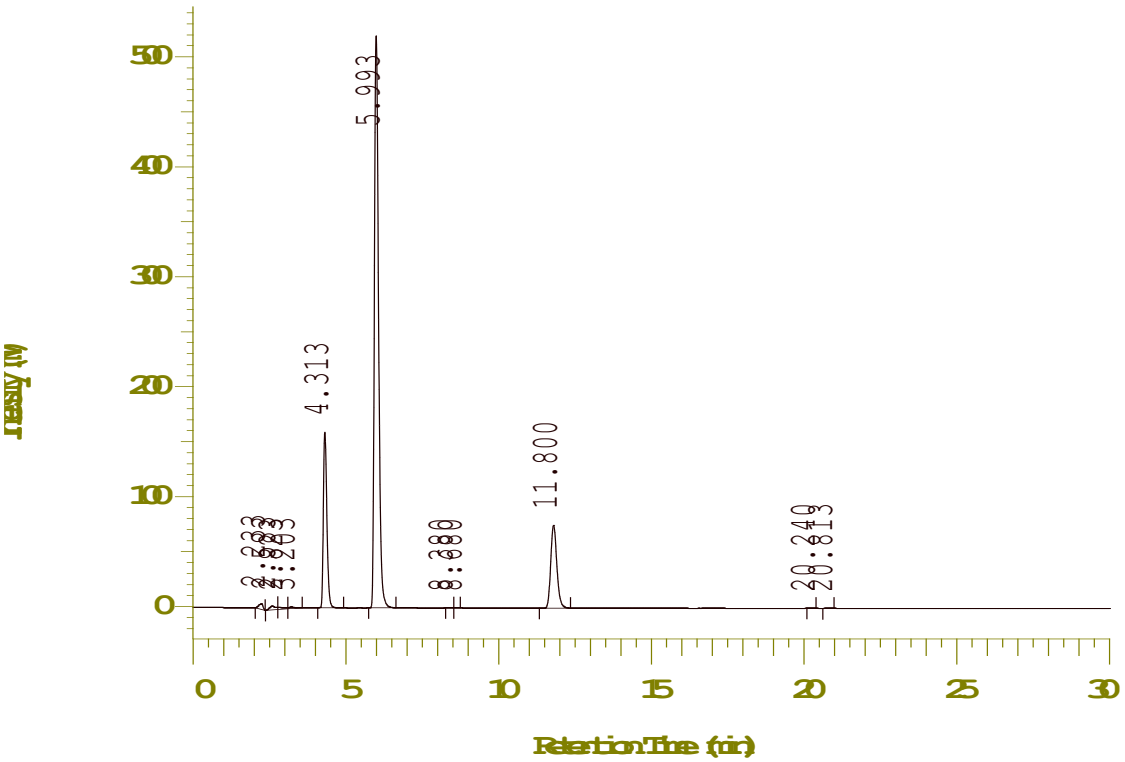


Figure 4.2: A chromatogram of a sample containing K (kaempferol) with a concentration of 1 mg/ml. The mobile phase MP consisted of 10 mM H_3PO_4: Me: ACN (50:25:25).

The peak for K is found at 5.993 min. The peak at 4.313 min coincides with the peak for Q as seen in figure 4.3 below. This shows that there is possibly trace amounts of Q present in K. This could be possible as it has been seen that Q in the body is conjugated to K during some studies, leaving the possibility that this is also possible outside of the body (Breinholt *et al.*, 2002). The peak at 11.800 min may possibly coincide with C, but has a shorter Rt than C as shown in figure 4.1. This shows that the K standard contains several unidentified compounds in trace amounts, including Q.

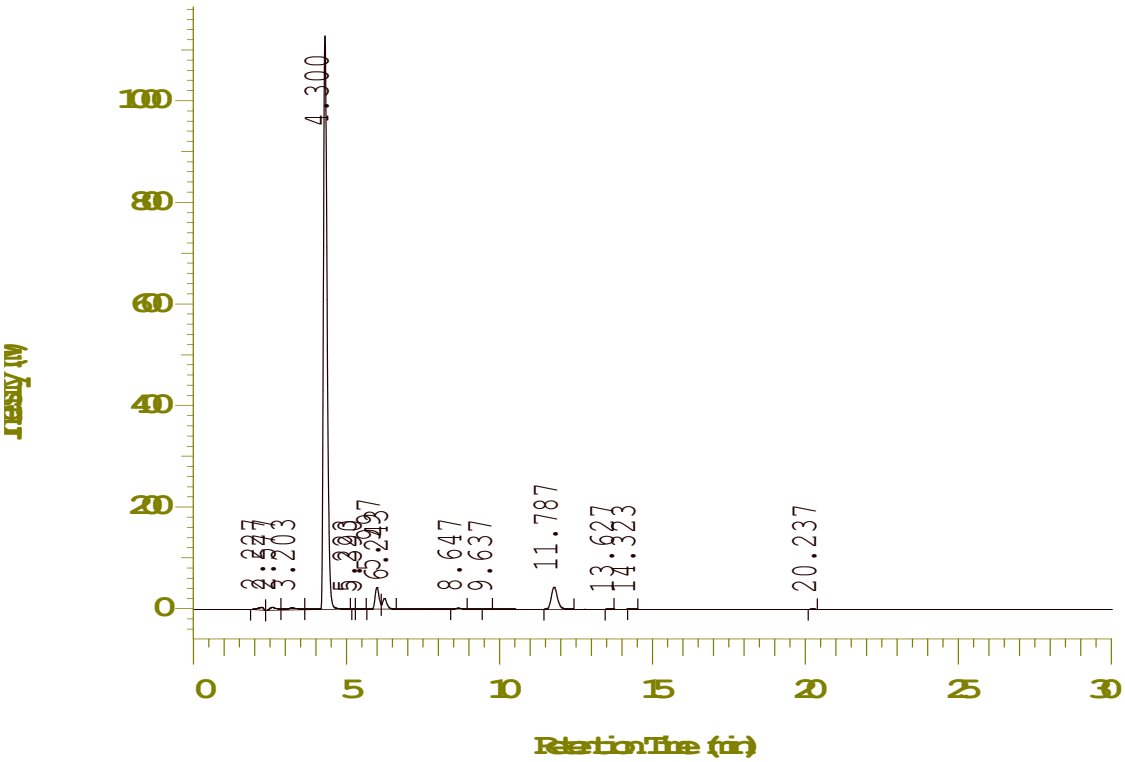


Figure 4.3: A chromatogram of a sample containing Q (quercetin) with a concentration of 1 mg/ml. The MP consisted of 10 mM H_3PO_4: Me: ACN (50:25:25).

In figure 4.3 the peak for Q is found at 4.300 min. The small peak at 5.997 min coincides with the peak for K as can be seen in figure 4.2. The peak at 11.787 min coincides with the peak for C as in figure 4.2. This shows that Q has very low trace amounts of the other compounds tested in this study (C and K) as well as several unidentified compounds.

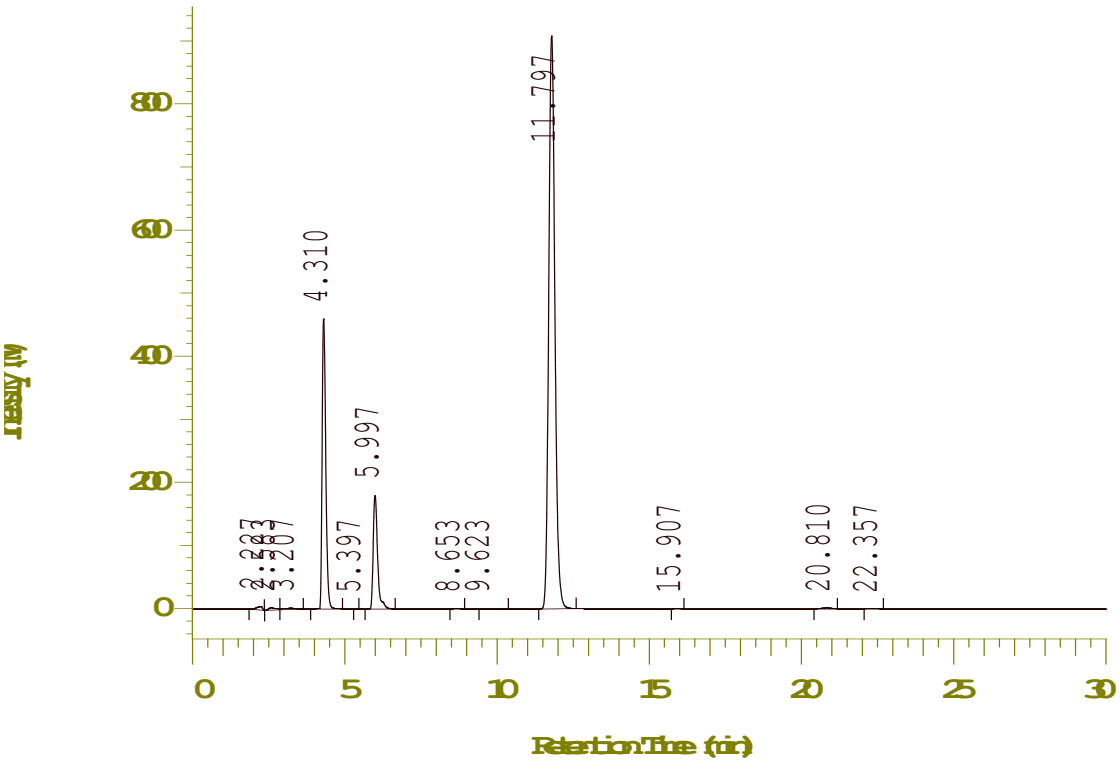


Figure 4.4: A chromatogram of a sample containing M (the mixture of single standards C, K and Q) from concentrations of 1 mg/ml. The MP consisted of 10 mM H_3PO_4: Me: ACN (50:25:25).

In figure 4.4 the peaks and retention times of all three analytes are shown. The Rt of Q is shown at 4.310 min, which corresponds to the values shown in figure 4.2 and 4.3. The Rt of K is shown at 5.997 min, which corresponds to the values shown in figure 4.2 and 4.3. The Rt of C is found at 11.797 min, which is slightly shorter than that of C in figure 4.1. It does, however, coincide with the peaks for C in figure 4.2 and 4.3. Other trace amounts of unidentified compounds can also be seen.

4.4 Discussion

The results shown for the chromatographic separation of standards in section 4.3 show that C, K and Q are clearly separated and identified. K and Q are also shown to contain trace amounts of one another, as was also shown by TLC in section 3.5 and 3.6 for the Q standard. This method was validated as can be seen in the following sections. During analysis of extract samples, retention times were compared to that of the standards to identify compounds (C, K and Q). These results are given in section 5.4.

4.5 Validation

4.5.1 Introduction

Validation can be described as verifying if the analytical purpose the method used for has been achieved. This entails obtaining analytical results with an acceptable error margin (Green, 1996; Taverniers *et al.*, 2010). The International Organization for Standardization (ISO) defines this as "confirmation by examination and provision of objective evidence that the particular requirements of a specified intended use are fulfilled (Anon, 2005)." If a method has been found to be useful for its intended purpose, the requirement is that the performance characteristics which the method states it is able to achieve needs to be demonstrated. The most common aspects addressed during validation are applicability, calibration, limit of detection, limit of quantification, operating range, precision, recovery, ruggedness, selectivity and sensitivity (Green, 1996; Magnusson & Ornemark, 2014). This is focussed on a defined method protocol, a defined concentration range for the analyte in use and a specified test material (Taverniers *et al.*, 2010; Thompson *et al.*, 2002). When this analytical purpose has been met, it shows that analytical results with an acceptable accuracy standard has been achieved (Taverniers *et al.*, 2010). As this study is more focused on the determination of presence of certain flavonoids, rather than quantity, only specific requirements of validation will be discussed as is deemed necessary.

4.5.2 Selectivity/Specificity

Selectivity is defined as the extent to which the accurate quantification of the analyte in the presence of other components that could cause interference (when in mixtures or matrices) during use of the specific method is possible (Vessman *et al.*, 2001). This helps in the identification of interfering compounds that could play a role during analysis. Thus, to say a method is selective, the analyst should be able to clearly state that the analyte being tested for can be clearly defined from the most pronounced interferents. To identify this, a single blank is usually compared to a blank that has been spiked (Thompson *et al.*, 2002). When these interferences are measured, it could cause a bias as it increases or decreases the signal towards the measurand. Effects that play a role are proportional/rotational and translational/fixed effect which is referred to as background/baseline interference (Magnusson & Ornemark, 2014).

4.5.3 Linearity and working range

The working range is defined as “the interval over which the method provides results with an acceptable uncertainty (Magnusson & Ornemark, 2014).” During validation, when assessing instrument working range, it becomes necessary to a) confirm the limit of quantification’s (LOQ) relationship with the upper end of the working range (which is the instruments response called linearity), b) show instrument working range and the interval stated in the scope of the method are compatible and c) verify that the procedure used for instrument calibration is adequate (Magnusson & Ornemark, 2014). Linearity is of importance as it addresses any problems that could have occurred during calibration of the instruments in use, taking into account the working range of the method (Magnusson & Ornemark, 2014). Calibration affects the strategy to ensure optimal development of the procedure. Thus it becomes necessary to ask if the calibration is a) linear, b) passes through the origin if a regression graph is drawn and c) shows that it stays unaffected by the matrix of the material being tested (Thompson *et al.*, 2002).

Thus, after calibration a regression of the data points is drawn using a range of specified concentrations. If there is no guidance for this specified, the general course is (Magnusson & Ornemark, 2014; Thompson *et al.*, 2002):

- There should be at least six or more standards of differing concentrations used to obtain data points for the curve.
- Using a specified concentration range, these calibration standards should be evenly spaced (Magnusson & Ornemark, 2014).
- The specified range should encompass 0-150% or 50-150% of the concentrations that can be encountered during analysis (Green, 1996).
- Calibration standards are to be run in duplicate (minimum) or triplicate in a random order.
- An initial visual inspection of the working range response curve is done.
- The regression line should have an R^2 value > 0.99 (for example) to ensure the confidence interval is met. The usual R^2 value is usually regarded as > 0.999. When considering impurities, the R^2 values is considered sufficient if it is > 0.98. R^2 is the coefficient of determination, representing the proportion of variance of the predicted variable (y-axis), from the independent variable (x-axis) which is the concentration (Green, 1996).

This data helps the analyst determine if the suggested calibration procedure is appropriate in response to the curve obtained.

4.5.4 Precision

Precision is defined as the relative closeness/similarity between individual test results obtained under the same specified conditions. Simplified, it can be asked, how close are the results to one another (Deldossi & Zappa, 2009; Magnusson & Ornemark, 2014). Precision is expressed using the statistical parameters describing the spread of results, called standard deviation (SD) and relative standard deviation (RSD). This is calculated from the results obtained after replicate measurements have been carried out on a suitable matrice with specified conditions (Magnusson & Ornemark, 2014). The two sets of conditions that are relevant are a) precision obtained under repeatability conditions (this compares variations seen doing a single run as expectation 0, being the most frequent result, versus the SD seen) and b) precision obtained during run-to-run conditions. This study focussed on repeatability, which is the measure of variability in results. Measurements are done by one analyst using the same equipment over a specified period (short time-scale). Reproducibility, measuring the variability between labs, was not done in this study. The goal should be to determine typical variability, rather than minimum variability (Magnusson & Ornemark, 2014).

The test conditions specified must resemble the normal conditions during normal laboratory routine during use of the method as to ensure the precision values adhere to these conditions. This includes variations in batches used, analysts and the instruments in use (Thompson *et al.*, 2002). A minimum impurity precision RSD% of 5% is sufficient in most cases for instrumentation values obtained during analysis as the confidence interval should be 95% (De Bievre *et al.*, 1998; Green, 1996; Taverniers *et al.*, 2010). If the compound is considered as an impurity, it should be present in <10%. The replicates used generally fall between 6 and 15 for each material undergoing analysis (Magnusson & Ornemark, 2014).

Precision is dependent on a range of analyte concentrations decided on beforehand (Magnusson & Ornemark, 2014). When this is done as a qualitative measure, it is done on an identification scale with a “cut-off” concentration where a simple “yes”/”no” is indicative of the presence at that concentration. For these measurements, SD or RSD is difficult to use and is thus expressed as true and false positive or negative results. “Cutt-off” concentration is established by determining the number of positives and negatives at different levels of concentrations (Magnusson & Ornemark, 2014).

4.5.5 Results and discussion

For validation purposes, The HPLC system used was a Hitachi Chromaster chromatographic system. The system consists of a 5410 UV-detector, an autosampler (5260) with a sample temperature controller and a solvent delivery module (5160). The column used for analysis was a Kinetex® EVO C_{18} column (250 mm x 4.6 mm; 5 µm particle size and 100 Å pore size).

4.5.5.1 Specificity

It can be seen from the chromatograms provided in section 4.4 in figure 4.1, 4.2, 4.3 and 4.4 that this method is specific as the samples peaks (figure 4.1, 4.2 and 4.3) can be clearly identified as the individual compounds tested for in the mixed standard sample (figure 4.4).

4.5.5.2 Linearity and range

For each of the three standards, a 1 mg/ml stock solution was prepared. Serial dilutions were prepared to yield solutions with concentrations of: 500 µg/ml, 100 µg/ml, 50 µg/ml, 10 µg/ml, 5 µg/ml and 1 ug/ml. Table 4.2 shows the working range concentrations and the individual average AUC values for C, K and Q.

Table 4.2: The working range concentrations and their average AUC used to determine linearity.

	Average AUC		
µg/ml	**Q**	**K**	**C**
1	11054	12862	36073
5	63295	62254	203211
10	131172	126590	425803
50	619059	578999	1982581
100	1292297	1209138	4142810
500	4941317	4646336	15810625
1000	9754154	9257515	29940399

The linearity for each individual compound is shown in figures 4.5, 4.6 and 4.7, respectively.

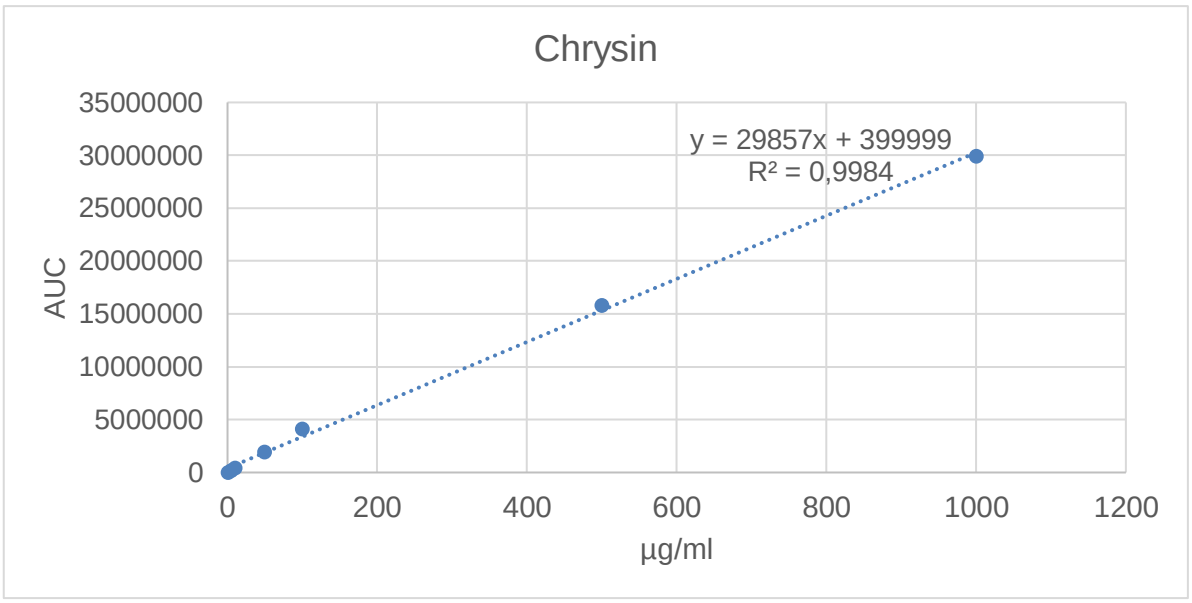


Figure 4.5: The linearity for chrysin when average AUC values of the different concentrations are plotted on a graph.

Figure 4.5 shows that C has a R^2 value of 0.998 4. It does fall in the > 0.98 range as specified for impurities as this is a test done for analysis of plant material. This shows that calibration is acceptable when linearity and working range is taken into consideration for C. The y = mx + c equation for the regression line is y = 29 857x + 399 999.

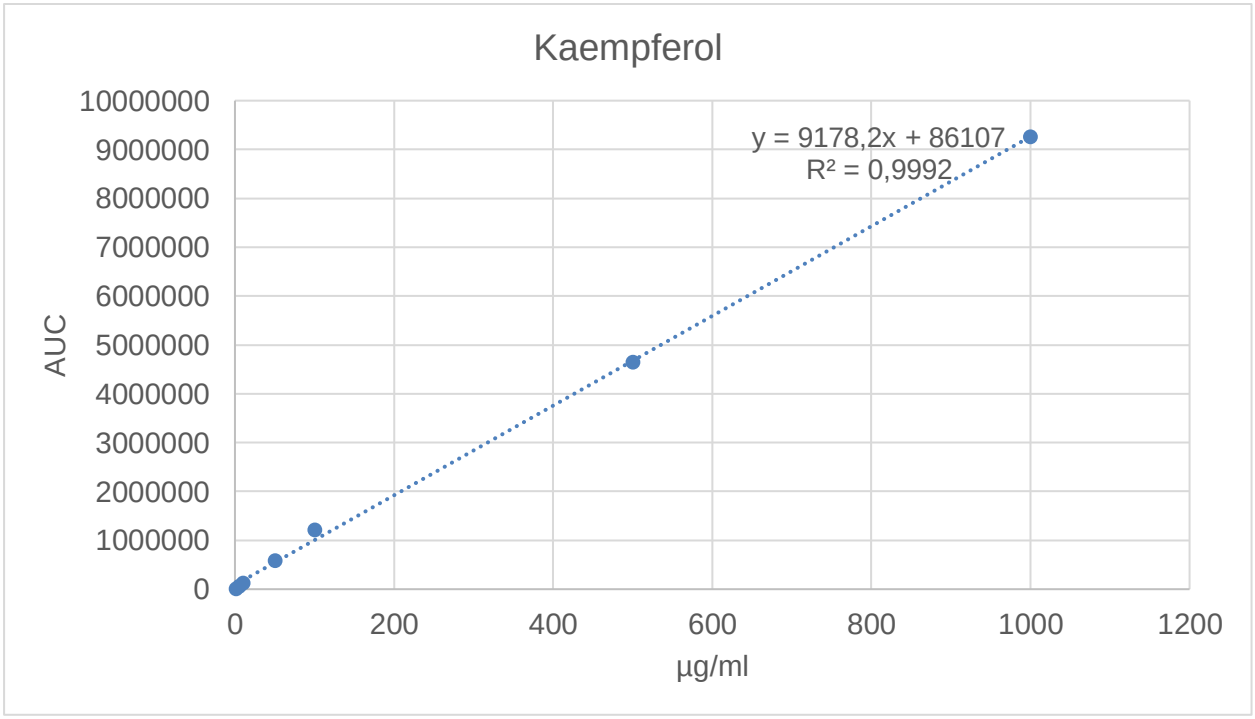


Figure 4.6: The linearity for kaempferol when average AUC values of the different concentrations are plotted on a graph.

Figure 4.6 shows that K has a R^2 value of 0.999 2. This falls in the ideal range which is generally accepted. This shows that calibration is acceptable when linearity and working range is taken into consideration for K. The y = mx + c equation is y = 9 178.2x + 86 107.

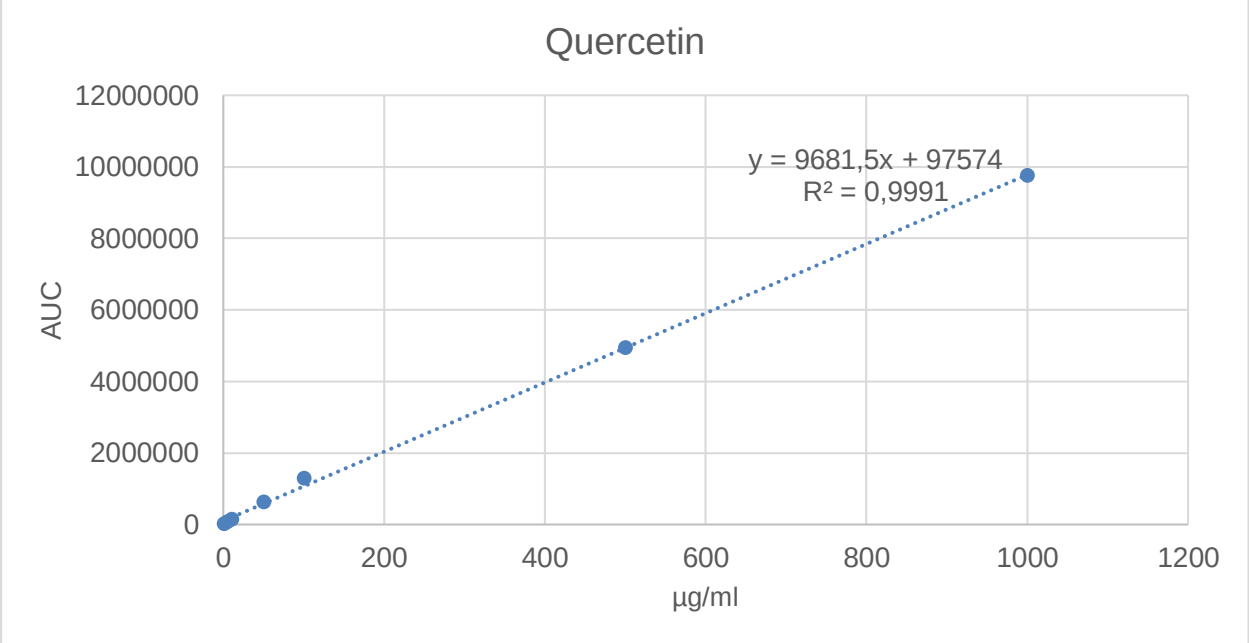


Figure 4.7: The linearity for quercetin when average AUC values of the different concentrations are plotted on a graph.

Figure 4.7 shows that K has a R^2 value of 0.999 1. This falls in the ideal range which is generally accepted. This shows that calibration is acceptable when linearity and working range is taken into consideration for Q. The y = mx + c equation for the regression line is y = 9 681.5x + 97 574.

For these graphs it can be seen that linearity is achieved over the working range for the three compounds under investigation.

4.5.5.3 Precision

To test for precision, it was decided to use a very low concentration of the analytes as there are no guidelines for validation when working with plant material. Since plant material shows a great variance which is difficult to predict, in this study, our goal was to keep our standards as close to the suggested standards as specified by EURACHEM (Magnusson & Ornemark, 2014). The concentration decided on for repeatability studies was 5 µg/ml. The results show that the RSD% values were smaller than 10%. In table 4.3 the repeatability values for the concentration of 5 µg/ml is shown:

Table 4.3: The repeatability results for 5 µg/ml to validate precision.

	AUC		
µg/ml	**Q**	**K**	**C**
5	64071.000	64204.000	207025.000
5	65233.000	62662.000	204158.000
5	63404.000	62801.000	204190.000
5	62670.000	61488.000	200383.000
5	62372.000	60761.000	202535.000
5	62021.000	61608.000	200976.000
Average	63295.167	62254.000	203211.167
STDEV-P	1098.145	1118.130	2230.532
%RSD	1.735	1.796	1.098

From table 4.3 it can be seen that six values were obtained for repeatability studies at a concentration of 5 µg/ml. At this low concentration, repeatability for C, K and Q values were below 2%, with Q being 1.735%, K being 1.796% and C being 1.098%. Since the general accepted rule is that variance worsens at lower concentrations, it is expected that the repeatability at higher concentrations should be even better. This shows that the repeatability for this method is acceptable.

4.6 Conclusion

From the above, it is evident that the method developed in this study is more qualitative than quantative, as the validation results are acceptable for identification purposes, as a full validation was not done which considers limit of detection (LOD) and LOQ which are necessary for quantative studies. This method developed is deemed useful for identification purposes only and not quantifying concentrations. For this study, it was deemed acceptable for analysis of the extractions discussed in chapter 5.

CHAPTER 5 EXTRACTION

5.1 General background

No experimentation on the different compounds in plants can begin without an extraction. Nor can any analysis be performed on high performance liquid chromatography (HPLC) without an extract that has been obtained containing the desired components of the plant material. The basic definition of extraction is the concentrated active medicinal portions of plants from plant portions as raw materials using a selected solvent and a standard procedure. This procedure describes the separation of the analyte between two immiscible phases usually consisting of an oil or water phase. Between these two phases, a distribution coefficient can be calculated. The final goal of extraction is thus the separation of the soluble metabolites of the plant material from the insoluble plant material (Azwanida, 2015).

5.1.1 Chemical and physical aspects that influence extraction

Classic extraction generally uses different solvents like acetone, ethanol, methanol, ethyl acetate or any of these in combination with water ethyl acetate or steam distillation. Working according to the principle that a polar product is soluble in a polar solvent (for example methanol [Me], ethanol [E] and dimethyl formamide) and the same being true for nonpolar products (examples being hexane, cyclohexane, benzene and toluene [T]), it enables us to determine which solvents to use. As polarity of the natural products increases, so does solubility (Gupta *et al.*, 2012; Jadhav *et al.*, 2009). The main purpose of extraction is to separate the soluble components of the sample from the insoluble components (Azwanida, 2015). Table 5.1 gives more information on the polarities of different solvents used for extraction and the specific plant chemical groups they are used for (Gupta *et al.*, 2012):

Table 5.1: Solvents, their polarities and chemical properties for specific chemical class extractions in plants (Gupta *et al*., 2012; Hansen *et al*., 2012e; Kaufmann & Christen, 2002; Mandal *et al*., 2007; Moldoveanu & David, 2012b).

Polarity	Solvent (and solvent type)	Boiling Point (°C)	Polarity index	Dielectric constant (Ɛ')	Dissipation factor (tan δ)	Chemical class to be extracted

Low	n-Hexane (Non-polar)	69	0.1	1.9	0.02	Fats Waxes
	Cyclohexane (Non-polar)	81	0.2	18.5	-	Fats Waxes
	Diethyl ether (Proton accepting)	35	2.9	4.34	-	Aglycones Alkaloids **Coumarins** **Fatty acids** Terpenoids
	Dichloromethane (Dipolar)	40	3.1	8.9	-	Aglycones Alkaloids Terpenoids
	Chloroform[b] (Proton donating)	61	4.1	4.81	-	Aglycones Alkaloids Flavonoids Terpenoids
	Ethyl acetate (Dipolar)	77	4.4	6.02	-	Aglycones Alkaloids Glycosides
	Acetone	56.53	5.1	20.7	-	Aglycones Alkaloids Flavonols
Medium	Ethanol	78.5	4.3	24.6	0.941	Alkaloids Flavonol **Polyacetylenes[a]** Polyphenols **Propolis[a]** **Sterols[a]** Tannins Terpenoids
High	Methanol	64.7	5.1	32.7	0.856	Amino acids Anthocyanins

						Flavones **Lactones[a]** **Phenones[a]** Polyphenols **Quassinoids[a]** Saponins Tannins Terpenoids **Totarol[a]** **Xanthoxyllines[a]**
	Water	100	10.2	77.46	9.889	Amino acids Anthocyanins **Lectins[a]** **Polypeptides[a]** Saponins **Starches[a]** Sugars Tannins Terpenoids

[a]Chemical classes in bold can be obtained using one solvent.

[b]Toxic, avoid if possible.

In table 5.1, the boiling point is of importance as this shows at what point the energy supplied to the solvent is high enough to cause it to evaporate (move into the gas phase) and can cause the solvent to lose its solubility capability as temperature rises. When the boiling point of a liquid is reached, the external pressure from the atmosphere and the vapour pressure is equal (Kotz *et al.*, 2012).

The polarity index describes each solvents polarity with solvents with a higher polarity index having a higher polarity themselves, making them more soluble as mentioned above (Gupta *et al.*, 2012; Jadhav *et al.*, 2009).

As solubility is dependent on the ability of the dissolved molecules to dissociate between its different ions, it is influenced by a solvent's dielectric constant. The dielectric constant describes a medium's capability to store electrical energy in an electrical field, which, in solvents influences

its ability to dissociate a molecule into its polar ions. If the compound is polar, it should have an electric dipole moment. In liquid form this is an indication of the polarity which influences solubility of the specific solvent (in bulk) used relative to the vacuum in which the extraction takes place. This means that the higher the dielectric constant is, the more polar the medium and the greater its solubility is (Moldoveanu & David, 2012a), affecting the polarity index of the solvent. As the dielectric constant also describes the induction capability of the solvent to overcome the electrostatic forces between a molecule's polar parts, it is also used in determining the solubility of solvents in their efficiency to dissociate the molecules of a compound (being bound by covalent, ionic, hydrogen bound or van der Waal's forces, depending on the molecule being a strong or weak acid or base). When applying heat, the dielectric constant starts lowering (as the spaces between molecules start increasing, making it harder to overcome the electrostatic forces between molecules), meaning polarity and solubility also start to decrease (showing the influence of boiling point) (Halliday & Resnick, 2011).

Before dissociation, there is not a complete polar alignment (meaning positive ends do not face the same way, as is true for negative parts as well), leading to the atoms constant bumping each other because of random thermal movement. As temperature increases (because of application of an external electric field), this thermal movement becomes less randomised as energy also rises, causing dipole rotation (meaning all the molecule's dipoles undergo realignment because of the constantly changing electric field) (Mandal *et al.*, 2007). Alignment produces an electric field with a smaller magnitude directly opposite to that of the field in which it is applied. This leads to the conclusion that if energy is applied to polar molecules in a medium (solvent), opposite alignment would occur, allowing the conduction of energy through it (Halliday & Resnick, 2011), causing the effect as can be seen in figure 5.1.

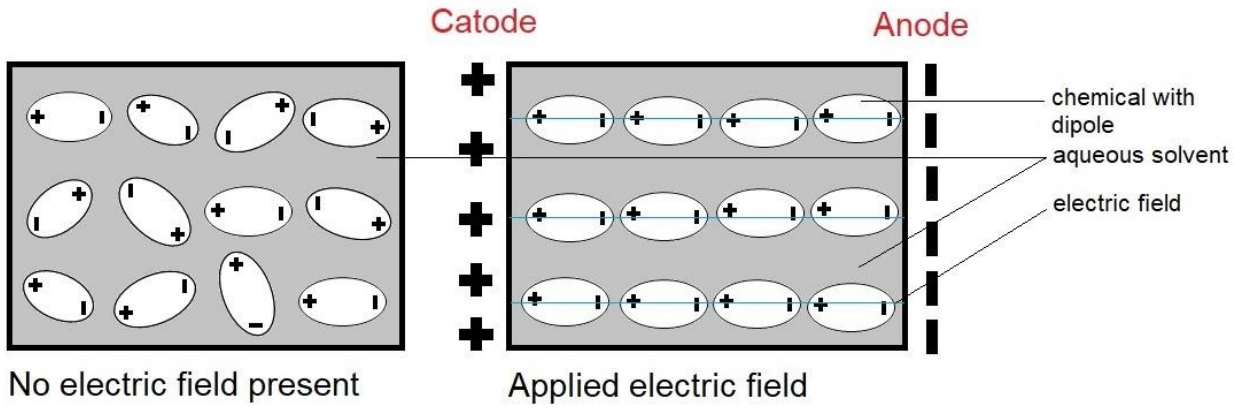


Figure 5.1: The dielectric effect shown when an electric field is applied.

If nonpolar atoms are induced, they acquire an electric dipole when induced by an external electric field. This is because of the extra energy applied that causes slight separation between the dipole areas of the atom, causing a definite positive vs negative pole (Halliday & Resnick, 2011). This is of more importance with certain extraction types as further discussed in microwave assisted extraction in 5.1.4.

The dissipation factor is the measure at which the solvent absorbs microwave energy which is passed on as heat to molecules in the surrounding area (Mandal *et al.*, 2007). This is described by equation 5.1:

$$\tan \delta = \frac{\varepsilon''}{\varepsilon'}$$

Eq. 5.1.

Equation 5.1: The dissipation factor equation (Mandal *et al*., 2007).

In equation 5.1, Tan δ is the dissipation factor which is calculated by dividing the dielectric loss represented by Ɛ'' (which in this case is an indication of the conversion from energy into heat [this can be seen as the loss of energy]) with the dielectric constant (Ɛ') (which measures the absorption of energy) (Mandal *et al.*, 2007).

Water will undergo more energy absorption than both ethanol and methanol because of higher Ɛ', but it still shows a lower heating efficiency than these solvents because of its lower tan δ value (Mandal *et al.*, 2007).

5.1.2 Objectives of extractions

The common objectives of extraction methods include (Azmir *et al.*, 2013):

- The extraction of bioactive compounds from the host sample.
- Analytical method selectivity improvement.
- Improvement of bioassay sensitivity by heightening targeted compound concentrations.
- Conversion of bioactive compounds into more reliable forms for separation and detection.
- Provision of a strong, reproducible method.

5.1.3 Sample preparation

Before extraction can begin, the plant material first undergoes preparation. This includes the roots, bark, leaves, flowers and fruit (Azwanida, 2015). Reasoning behind this is plant secondary metabolites consist of complex mixtures of substances with a wide variety of polarities and hydrophobicities. These metabolic groups are divided into low polar metabolites, which are the terpenoids and waxes, semi-polar metabolites, which are lipids, low-polar alkaloids and phenolic compounds (of which flavonoids are part) and highly polar metabolites, which are peptides, polar alkaloids, polar glycosides, proteins and saccharides (Romanik *et al.*, 2007). Preparation before extraction procedures can be seen as the freshness (indicating time since harvested, be it a day or a week), drying, grinding and any other processing procedure that can cause a change in the phytochemical molecular consistency of the material. Fresh plant samples tend to be more fragile than their dried samples counterpart, showing accelerated deterioration in comparison (Azwanida, 2015). It has to be taken into consideration that a loss in analyte fraction can occur during the sample preparation procedure, especially when the analyte amounts are very small. To determine the compound contents of the final extract in relation to the original tissue, percentage recovery is an important calculation (Romanik *et al.*, 2007). For this reason, optimisation of efficiency and efficacy has been focused upon. Efficiency can be described as the extraction yield and efficacy as the potency of the extraction (meaning the extracts measurable bioactivity or effectiveness in producing an effect) (Gupta *et al.*, 2012).

5.1.4 Extraction methods

Extraction methods can be classified into two different categories which can be described as traditional and modern extraction methods. Advantages of the modern extractions over traditional extractions include that less solvent is used during extraction, minimising sample degradation, as well as elimination of insoluble components from the extract with better sample yield; and also having faster extraction times (Azmir *et al.*, 2013; Gupta *et al.*, 2012). Traditional extraction methods include (Azmir *et al.*, 2013; Azwanida, 2015):

- Maceration
- Soxhlet Extraction
- Hydro distillation
- Water Bath Extraction (WBE)

The more modern extraction methods that have been developed include (Azmir *et al.*, 2013; Azwanida, 2015):

- Ultrasonic Extraction (USE)
- Microwave Assisted Extraction (MAE)
- Accelerated Solvent Extraction
- Supercritical Fluid Extraction
- Enzyme Assisted Extraction
- Pulsed-Electric Field Extraction

Only the extraction methods used in this study will be discussed in more detail, which is maceration (5.1.4.1), USE (5.1.4.2), WBE (5.1.4.3) and MAE (5.1.4.4).

5.1.4.1 Maceration

Maceration is a method used to destroy the cell walls of the sample so that the soluble component can be released. This is achieved by soaking the ground sample in a solvent (the menstruum) in a closed container at room temperature over a specified period of time. Frequent agitation is required for the method to be effective. Agitation helps extraction by increasing diffusion and bringing new solvent into contact with the sample while it removes concentrated solvent (Azmir *et al.*, 2013). Though this is a simple method, heat transfer still occurs via convection and conduction during the process, enhancing the procedure. When the specified time period is over, the contents of the container is filtrated by straining or pressing. Of all the extraction methods used, this is the most simplified method. Some of the biggest drawbacks found in using this method is that it uses a large amount of solvent and also produces a high amount of waste product (Azwanida, 2015).

5.1.4.2 Ultrasonic extraction

This is an extraction method that uses ultrasound to accomplish separation as ultrasonic waves cause compression and expansion. USE is divided into two separate phenomena, the one being diffusion of the ultrasonic waves through the vegetational cell walls of the sample material and release of the materials from the material as soon as these cell walls are destroyed (Cares *et al.*, 2010). The acoustic cavitation phenomenon (the production, growth and collapse of bubbles

formed by ultrasonic waves) occurs first, producing kinetic energy that heats the material it is projected into. Cavitation only occurs in liquids and liquid containing solids (such as plants), which improves the contact between the solid and liquid, thereby increasing permeability into and out of the plant material, increasing the mass transfer (Azmir *et al.*, 2013; Cares *et al.*, 2010). Vibration of these solids and liquids are increased (with the implosions of bubbles against the cell walls causing damage to its structural integrity, forming micro capillaries through which the solvent enters the plant material), causing diffusion out of the solid into the liquid (solvent) to speed up (Cares *et al.*, 2010).

The frequency used can range from 20 kHz to 2 000 kHz. This method works through a mechanical effect that enlarges the surface contact between the solvents and samples. This increases the permeability of the cell walls by disrupting it, thereby increasing transport of solvents into the sample, as well as quickening the release of compounds out of the sample (Azwanida, 2015). This method can be optimised by adjusting ultrasound application factors which are frequency, sonication power and extraction time and ultrasonic wave distribution (Biesaga, 2011). Benefits pertaining to the use of USE include a shorter extraction time as well as a smaller amount of solvent and energy use (Azmir *et al.*, 2013). It is a highly reproducible method with a smaller energy and temperature input and easier manipulation and tends to show better results for heat labile compounds (Biesaga, 2011; Gupta *et al.*, 2012). The only problem is that above 20 kHz, free radicals can form (Azwanida, 2015).

5.1.4.3 Water bath extraction

WBE, in this instance, was used to ensure heat transfer into the plant material at a constant temperature for the specified time period in which the extraction is completed with a constant rotation motion to ensure penetration of the plant material does occur. This is a method setup adopted and adjusted from a previous study done by Søltoft *et al.* (2009). In another study done by Liu and Zhu (2007), WBE was used at a relatively high temperature of 70 °C for a very short time of 11 minutes, compared to the two hour extractions studies performed at room temperature in this study. Søltoft *et al.* (2009) also stated that this method consumed more solvent in their study, compared to other extraction methods used. Out of a study done comparing WBE to USE for extraction of carnosic acid from rosemary, it was found that the same extraction quantity achieved in 3 hours using WBE could be obtained in 15 minutes using USE (Albu *et al.*, 2004).

5.1.4.4 Microwave assisted extraction

A microwave is an electromagnetic field. It works in the frequency range of 300 MHz to 300 GHz. On the electromagnetic spectrum they are found between X-ray and infra-red rays (Hansen *et al.*, 2012c). Two perpendicular fields, an electric and magnetic field, are used that create oscillation (Azmir *et al.*, 2013). Two mechanisms are at work during this time creating the oscillations, being ionic conduction and dipolar rotation. Ion conduction refers to electrophoresis transpiring, causing the ions to become charged and migrate under the influence of the applied electric field. Migration of the ions causes friction that causes the rise in heat in the system (Mandal *et al.*, 2007). Alignment of the electric fields causes the dipolar rotation on the molecules of the solvent and matrix material which possess a dipole moment. This applied energy causes the surrounding molecules to collide, releasing thermal energy into the solvent. The solvent itself does not absorb the energy, but rather conducts it into the plant materials moisture content. The moisture evaporates, building up pressure from the vapours inside the sample, breaking the cells of the cell walls and releasing its content into the solvent. The whole sample is heated simultaneously, in comparison to gradual heating taking place considering conventional methods (Kaufmann & Christen, 2002). MAE is a better method when the solvent is polar. The sample material absorbs the electromagnetic waves generated by the microwave that is converted into heat (Gupta *et al.*, 2012). The heating from the radiation (especially dipolar rotation) waves disrupt the weak H-bonds as heat is transferred into the sample which promotes solvent penetration as dissolved ions migration into the matrix increases. The higher the dielectric constant of the solvent, the more optimal is the heat generated (Gupta *et al.*, 2012; Kaufmann & Christen, 2002). This method is not as useful when the solvent is non-polar (Azwanida, 2015).

Literature states that the chance of thermal degradation is heightened using MAE, while the amount of solvent used and extraction time is decreased (Azwanida, 2015; Kaufmann & Christen, 2002). Losses are minimised as extraction is performed in a closed area, and degradation by heating time can be reduced if limits are applied to experimental conditions. Using MAE ensures that all the sample-fluid is heated which lets the extraction fluid reach an ideal temperature at a faster pace. Doing this ensures that a thermal gradient will not be reached as is done by conventional heating methods (Azmir *et al.*, 2013; Biesaga, 2011; Kaufmann & Christen, 2002).

5.2 Chemicals and apparatus

Table 5.2 describes the different chemicals and apparatus that were used to complete extractions for analysis on HPLC:

Table 5.2: The different chemicals, apparatus, plants and other materials used for extractions.

Chemicals, apparatus, plants and materials used for extractions:			
Item:	**Grade:**	**Batch number:**	**Company:**
Chemicals:			
Methanol (CH_3OH)	Gradient grade for liquid chromatography	I0930107 803	Merck©
HPLC water	Milli-Q®		
Appliances:			
Blender	Desc: Mellerware table blender Model: Sylvio jar blender Type: 62200A Voltage: 220-240 V ~ 50/60 Hz Wattage: 550 W		Mellerware©
Hot plate	Model: 105 Desc: Magnetic stirrer with heating Voltage: 230 V ~ 50 Hz Wattage: 550 W		Labotec©
Warm water bath	Type: RST II Nr: 28574 Wattage: 1 025 W Voltage: 220 V		Retostat©
Ultrasonic cleaner	Scientech Model: 705		Labotec©

	Desc: 25.0 L Ultrasonic bath with timer function Voltage: 230 V ~ 50 Hz Wattage: 500 W		
Microwave	DEFY Model: DMO 343 Desc: Microwave oven with grill **Input:** Voltage: 230 V ~ 50 Hz Wattage: 1 500 W **Output:** Voltage: 2 450 MHz Wattage: 1 000 W	DMO343AB10332490	DEFY©
Stove	Defy Model: Slimline 600 hob Voltage: 220-240 V ~ 50 Hz Wattage: 5 500 W Hot plate Wattage: 1 000 W		DEFY©
Plant specimens and edible products:			
Yellow onions	1 kg class 1 locally grown		Freshmark©
Red onions	1 kg class 1 locally grown		Freshmark©
Broccoli	Class 1 locally grown		Freshmark©
Butter			
Margarine	Blossom®		

Teas			
Bergemont	Earl grey® 2 g tea bag		Lipton©
Rooibos	Freshpak®		
English tea	Five Roses®		
Kilimanjaro tea English breakfast®			
Dilmah Ceylon tea from Sri Lanka®			

5.3 Procedure and method development

Before the beginning of any procedure, it is of utmost importance to ensure that all glassware, apparatus, appliances, chemicals and plant specimens are clean. With the plant specimens, this was ensured by the use of normal tap water, followed by distilled water and lastly HPLC grade water to ensure that all organic contaminants have been removed. After this, standard preparation procedure could begin.

5.3.1 Preparation

5.3.1.1 Onions (red and yellow) - General procedure

The general procedure for preparation followed for normal fresh onions, frozen onions and cooked onions included the following steps:

- The dry outer layer of the onion was removed so as to expose only the inner layers.
- The top (blossom end) and bottom (root end) part of the onion was removed as to ensure only the base area of all layers were used as shown in figure 5.2.

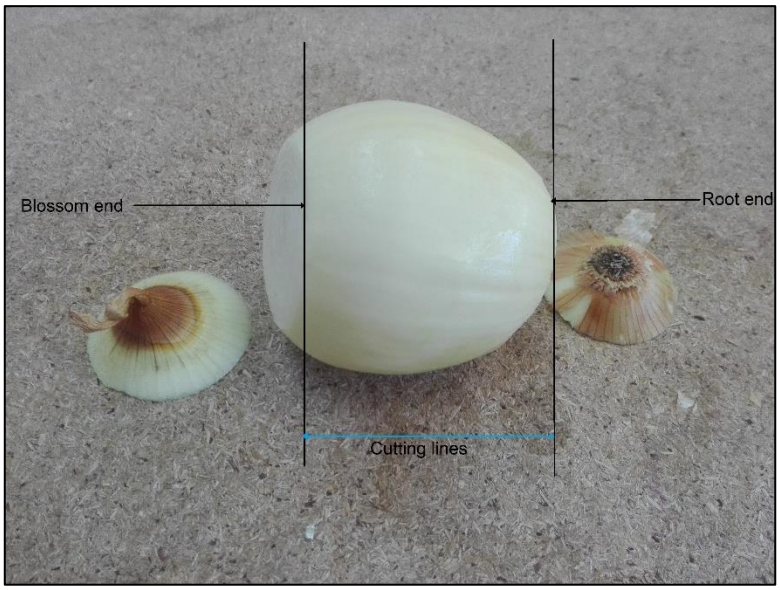


Figure 5.2: Part of the onion used for extractions after first cuts.

- The onion area used was then washed as described in the procedure above (5.3).
- After washing, the onion was further cut to test three different area sizes. This was horizontal round cuts (described as “whole” onions), horizontal round cuts that had been diced (described as cut onions) and diced onions that had been blended for a period of 2 min set at speed 4 (described as blended onions). Figure 5.3 shows the difference between these three cuts used.

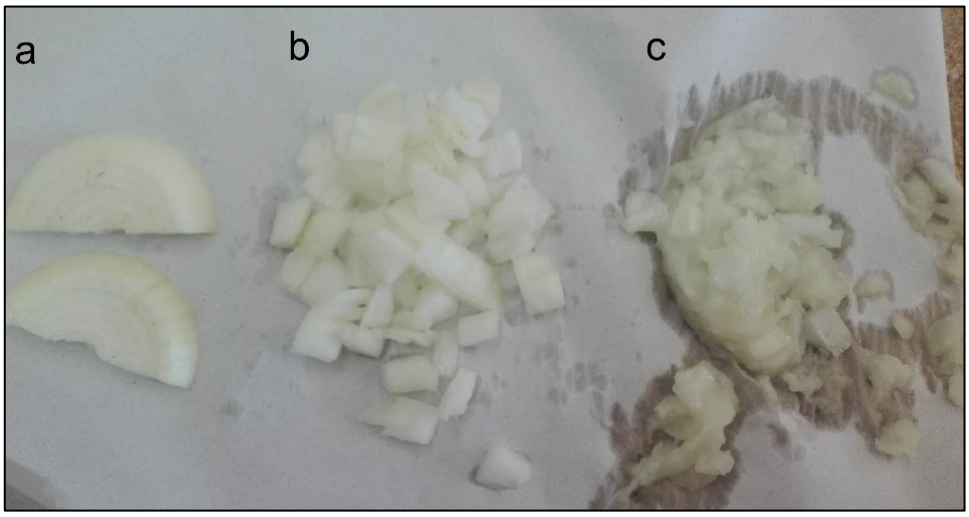


Figure 5.3: The three different cuts used with onion preparation, being (a) whole, (b) cut and (c) blended.

- Once the cutting had been done, continuation with the extraction procedures could begin.

5.3.1.2 Broccoli – general procedure

The general procedure for preparation of broccoli for extraction included the following steps:

- The whole broccoli head was washed before further preparation following the procedure as described in section 5.3.
- All shrivelled, dried edges of the broccoli were cut off.
- Smaller pieces of the whole broccoli were used in the extractions.
- Some of the whole broccoli was blended for about 2 min with the speed set to 4, after which it was ready to be used for extractions.
- The florets part of the broccoli (meaning without the stems) were cut off which was also used for extractions.
- Some florets were blended for about 2 min at the speed set to 4, before being used as extractions. Figure 5.4 shows the different end products of broccoli after preparation.

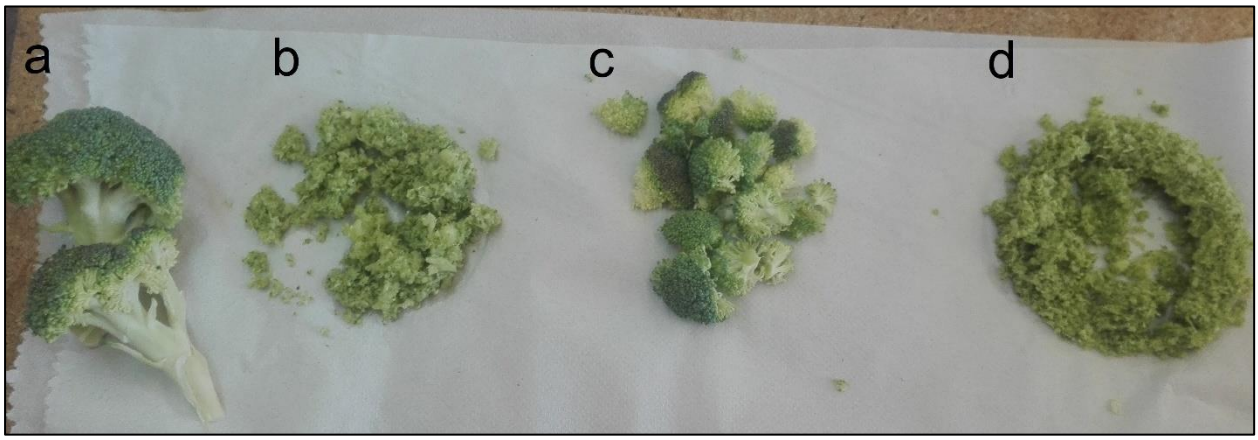


Figure 5.4: The four different types of broccoli preparations used for extractions, being (a) whole broccoli, (b) blended whole broccoli, (c) florets and (d) blended florets.

5.3.1.3 Preparation of the syringe for extraction

The following procedure in figure 5.5 shows the setup of the prefilter that was used with a syringe before extraction, which followed the following steps:

- The prefilter was set up as shown in figure 5.5.

- The filter was added onto the prefilter (creating the prefilter-filter system).
- The prefilter-filter system was added onto the syringe (creating the prefilter-filter-syringe system).
- This system was now ready to be used to take extracted samples for analysis in HPLC-vials.

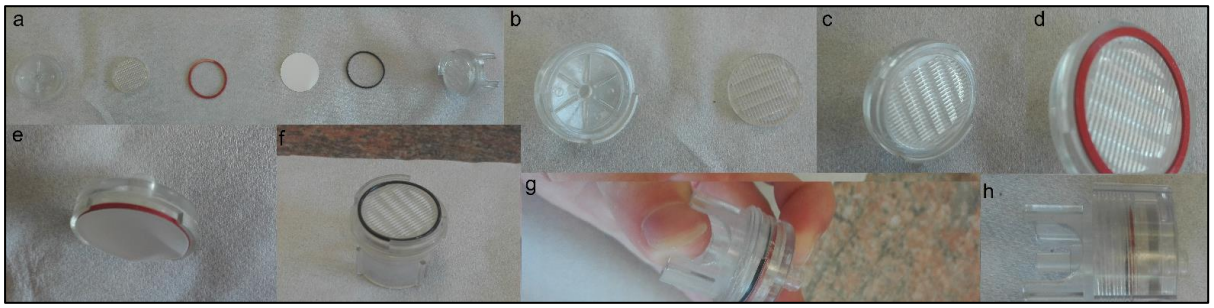


Figure 5.5: The setup of the prefilter.

The complete filter-prefilter-syringe system can be seen in figure 5.10 (5.3.8) as was used during sampling.

5.3.2 Maceration

The general maceration procedure followed after preparation of plant material was as follows:

- A clean glass beaker (50 ml) was used.
- Approximately 10 g of the prepared plant material was added into the glass beaker.
- 20 ml Me was added into the glass beaker.
- A magnetic stirrer was added into the glass beaker and the glass beaker was covered with parafilm.
- Using the magnetic stirring plate at its highest speed, the mixture in the glass beaker was left for two different time periods. Maceration was long extractions that lasted for eight hours (extracts were taken after 1 h, 4 hs and 8 hs as discussed in 5.3.8.).

Figure 5.6 shows the general setup used for maceration before extraction and while extraction was in progress:

Figure 5.6: General setup for maceration (a) before extraction and (b) during extraction.

5.3.3 Water bath

The following steps were followed for water bath extractions:

- The water bath's temperature was kept at room temperature (25 °C).
- After preparing the different plant materials for extraction (5.3.1.1. and 5.3.1.2.), plastic test tubes were filled with approximately 5 g plant material.
- 8 ml Me was added into the test tube.
- After closing, the test tube was shaken to ensure that the plant material had been completely submerged in Me.
- The test tube was inserted into one of the slots of the circulator in the water bath.
- The water bath was started and extraction samples were taken after 30 min, 60 min and 120 min as discussed in 5.3.8. The water bath's circulatory system ensured constant movement of the liquid in the extraction sample.
- Before reinserting the test tube in the water bath, an equivalent quantity Me taken as sample was used to refill the test tube to ensure enough solvent was available for the next extraction.

Figure 5.7 shows the water bath and a test tube used for extractions:

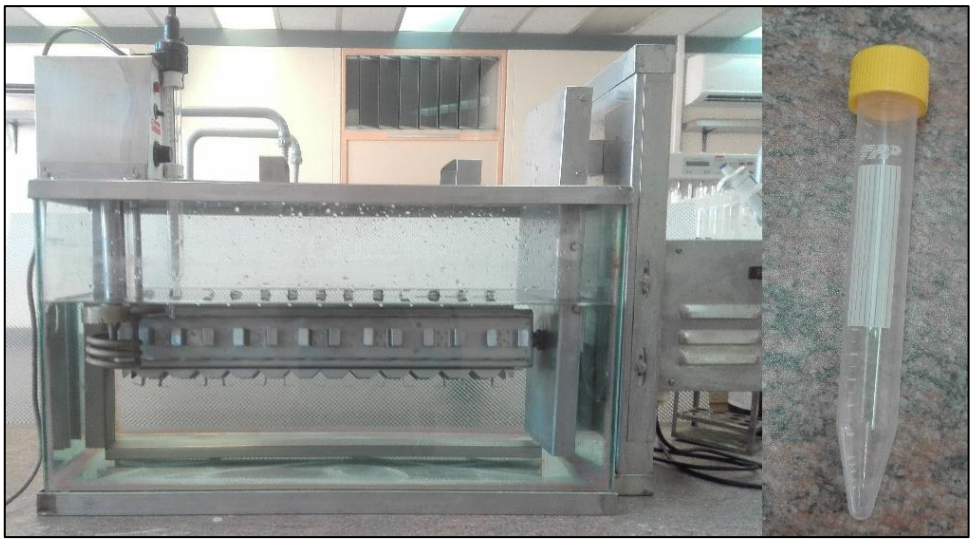


Figure 5.7: The water bath used for extractions (a) and a test tube which is to be inserted into one of the water baths circulator slots (b).

5.3.4 Ultrasonication

For USE, the following steps were followed:

- As in the case of WBE, after preparing the different plant materials for extraction (5.3.1.1 and 5.3.1.2), plastic test tubes were filled with approximately 5 g plant material.
- 8 ml Me was added into the test tube.
- After closing, the test tube was shaken to ensure that the plant material had been completely submerged in Me.
- The test tubes were placed into the ultrasonicator.
- The ultrasonicator is set to room temperature and the power to high.
- Sample extractions were taken after 30 min, 60 min and 120 min as discussed in 5.3.8. As in the case of WBE, the same equivalent Me used for sampling was used to refill the vial.

Figure 5.8 shows the ultrasonicator used for USE.

Figure 5.8: The ultrasonicator used for extractions.

5.3.5 Microwave assisted extraction (MAE)

The steps for MAE were as follow:

- The general preparation procedure for plant extracts were followed as discussed in section 5.3.1.1 and 5.3.1.2.
- The plant material was placed into a glass beaker.
- 20 ml Me was added and the beaker swirled to ensure that the plant material had been completely soaked and submerged in the Me.
- The glass beaker was left open (as the heat caused the Me to evaporate). To minimise the loss of evaporation, a plastic cup was placed over the beaker.
- The power was set to either 60% (600 W) or 80% (800 W) power. The MAE process was started.
- Samples were taken using the syringe system (5.3.1.3) as described in 5.3.8. This happened after 10 s, 30 s and 60 s.

5.3.6 Cooked

Steps for cooked samples were as follow:

- The general preparation for plant material was followed as discussed in 5.3.1.1 and 5.3.1.2.
- Approximately 10 g of the plant material was added into a 100 ml Erlenmeyer flask. The larger bottom and smaller neck was to compensate for the loss of solvent caused by heating, leading to evaporation.
- 30 ml HPLC H_2O was added into the flask.
- The warm plate was set to its highest heat as to warm up (about 5 min).
- The flask was covered with foil to prevent too much solvent loss.
- If too much solvent loss had occurred, 20 ml HPLC H_2O was added to the system. This happened on a regular basis, as to ensure that the sample did not cook dry or burnt, changing the chemical composition of the system.
- Since it was impossible to take samples of the plant material and continuing the cooking procedure as the plant material had to be macerated for sampling, decreasing the amount of plant material from the starting material and leading to inconsistent and variable amounts, the cooking procedure had to be repeated three times with the same quantity of plant material weighed off at the start of the procedure. This led to samples of the HPLC H_2O taken after 30 min (stopping this first cooking procedure), 60 min (stopping this second cooking procedure) and 120 min (stopping the third cooking procedure) as discussed in 5.3.8. Thus there was three different cooking procedures up until specific times.

The above method was to establish if any of the compounds analysed occurred in the H_2O, leaving the plant sample. The below method describes extraction to establish if any compounds were still in the plant material during or after cooking:

- After cooking, the plant material was removed from the cooking water.
- The plant material was pressed dry between lab paper.
- As soon as the water had been removed, the plant material was added into a 50 ml glass beaker.
- Further steps were followed as discussed in section 6.3.2 for maceration.
- Sampling was done according to the steps in 6.3.8.

Figure 5.9 describes the above process:

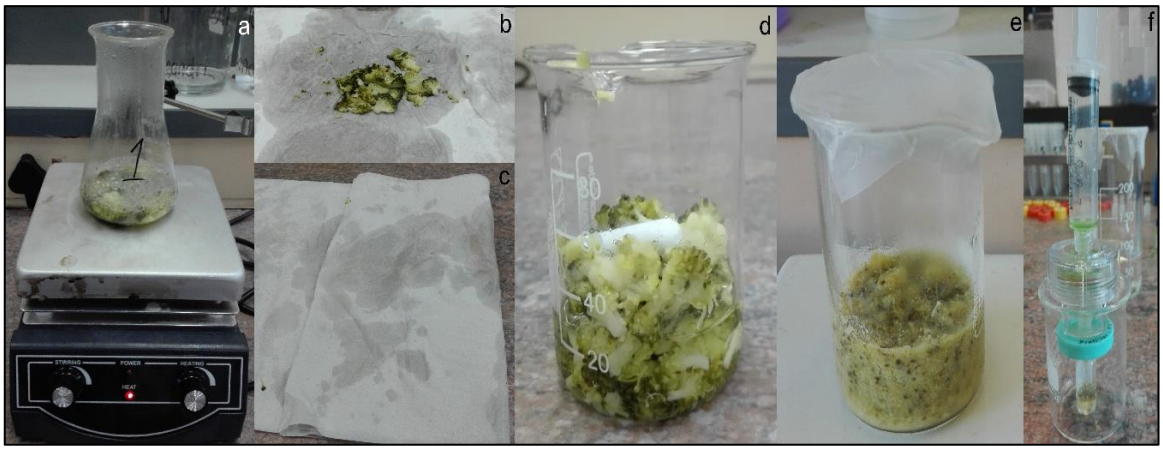


Figure 5.9: Cooked extractions procedure. The plant sample is cooked (a), after which it is placed on lab paper (b) and dried using the lab paper (c). The dried sample is added back into a glass beaker for maceration (d) and undergo maceration on the magnetic stirring plate (e). After maceration, a sample is taken for analysis (f).

5.3.7 Grilled onions

The following procedure describes the steps for extraction of grilled onions (red and yellow), which differ from the normally followed procedures:

Preparation:

Both the butter and margarine was melted in a frying pan on a hot plate at maximum power to test as standards and see if any of the compounds were present as well. 10 ml of the melted butter/margarine were diluted in 100 ml Me, which was filtrated. This filtrate was tested on HPLC as standards in conjunction to the normal C, K and Q standards.

Preparation:

- The top (blossom end) and bottom (roots end) parts of the onions were removed, as well as the outer dried layer.
- The onion was washed under tap water and diced.
- The hotplate was heated and the butter/margarine (10 ml) was heated until melted in a frying pan.

- As soon as the butter/margarine had been melted, the chopped onions (red or yellow) were added into the frying pan.
- The onions were continuously stirred with a spatula as to ensure that no plant samples became burnt. This continued for 3 min.
- As soon as the 3 min were up, the contents of the pan was emptied into a container for storing till extraction could occur.

Extraction:

- For extraction, the containers in which the grilled onions were kept, were placed in hot water to ensure that the contents (butter/margarine), stayed in liquid form.
- 10 g of the content was added into a glass beaker (50 ml).
- 20 ml Me was added as well as a magnetic stirrer.
- The top was covered with parafilm.
- Samples were taken after 30 min, 60 min and 120 min as discussed in 5.3.8.

5.3.8 Teas

The following steps describe the procedure for tea extractions:

- HPLC H_2O was boiled before extractions started.
- The tea bag was cut open.
- Half the content was weighed off in a glass beaker; and the other half into another glass beaker.
- Magnetic stirrers were added to both beakers.
- 20 ml boiled HPLC H_2O was added to the one beaker and 20 ml Me to the other.
- The glass beakers were covered with parafilm and placed on the magnetic stirring plate.
- Extract samples were taken after 30 min as discussed in 5.3.8.

5.3.9 Sampling of extractions

The following steps explain sampling of monsters for analysis:

- Using the syringe system as set up in 5.3.1.3, samples were taken.
- Before using the syringe system, the system was rinsed with HPLC-H_2O to ensure that there were no leakages.
- The plant extract, without adding too much of the bulk plant material into the syringe, was filtered through the prefilter-filter system (as to ensure that no particles were larger than 0.45 µm) into a HPLC glass vial.
- These extraction samples were then used for analysis on the HPLC system using the method developed in chapter 4.
- After sampling, the syringe-prefilter-filter system was rinsed before the next extraction. A new system was set up before a different method or plant material was used.

Figure 5.10 shows the process of sampling:

Figure 5.10: The process of sampling of extractions. The extraction is added into the syringe part of the syringe-prefilter-filter system, through which the sample is then filtrated into a HPLC vial by pushing down on the shaft of the syringe.

5.3.10 Analysis

All extract samples taken were analysed on HPLC using the method developed in chapter 4.

5.4 Results

The main goal of this study was establishment of the presence of the specific compounds. The quantitation of the compounds were only approximated to establish trends seen from the extraction process. It has to be taken into consideration that these extractions were not done in duplicate or triplicate, making it difficult to establish if the results obtained from extracts in the case of an outlier are due to laboratory mistakes, or can be considered as part of the identified trend.

5.4.1 Fresh plant samples

5.4.1.1 Presence analysis

Table 5.3 shows the presence of chrysin (C), (K) and (Q) in extractions done on fresh plant material of onions and broccoli:

Table 5.3: The presence of compounds chrysin, kaempferol and quercetin in fresh analysed plant samples.

Presence									
	Extractions (Fresh)								
	Yellow onion								
	Maceration			Water Bath			Ultrasonication		
	1 hour	4 hour	8 hour	30 min	60 min	120 min	30 min	60 min	120 min
Whole	Q;K;C	Q;K;C	Q;K;C	Q;K;C	Q;K;C	Q;K;C	Q;K;C	Q;K;C	Q;K
Cut	Q;K;C	Q;K;C	Q;K;C	Q;K;C	Q;K;C	Q;K;C	Q;K;C	Q;K;C	Q;K;C
Blended	Q;K;C	Q;K;C	Q;K;C	Q;K;C	Q;K;C	Q;K;C	Q;K;C	Q;K;C	Q;K;C
	Red onion								
	Maceration			Water Bath			Ultrasonication		
	1 hour	4 hour	8 hour	30 min	60 min	120 min	30 min	60 min	120 min
Whole	Q;K;C	Q;K;C	Q;K;C	Q;K;C	Q;K;C	Q;K;C	Q;K;C	Q;K;C	Q;K;C
Cut	Q;K;C	Q;K;C	Q;K;C	Q;K;C	Q	Q;K;C	Q;K;C	Q;K;C	Q;K;C
Blended	Q;K;C	Q;K;C	Q;K;C	Q;K;C	Q;K;C	Q;K;C	Q;K;C	Q;K;C	Q;K;C
	Broccoli								
	Maceration			Water Bath			Ultrasonication		

	1 hour	4 hour	8 hour	30 min	60 min	120 min	30 min	60 min	120 min
Whole	Q;K;C	Q;K	Q;K;C	Q;K	Q;K;C		Q;K;C	Q;K;C	Q;K;C
Blended	Q;K	Q;K;C	Q;K;C	Q;K;C	K	K	Q;K;C	Q;K;C	Q;K
	Florets								
	Maceration			**Water Bath**			**Ultrasonication**		
	1 hour	4 hour	8 hour	30 min	60 min	120 min	30 min	60 min	120 min
Whole	Q;K;C	Q;K;C	Q;K;C	Q;K;C	Q;K;C	Q;K;C	K;C	K;C	K;Q
Blended	Q;C	Q;K;C	Q;K;C	Q;K;C	K;C	K;C	Q;K;C	Q;K	Q;K

In this table, quercetin is shown by Q, kaempferol by K and chrysin by C.

It is evident that all three compounds (C; K and Q) can be extracted from fresh plant material (yellow and red onions). With the broccoli samples, the best extraction method seemed to be USE and the least favoured extraction method WBE as this method does not seem to deliver consistent results. Results obtained for analysis of the florets samples seemed less inconsistent as the presence of C, K and Q were less random. The extraction method, considering presence of the chemical compounds tested for, being most effective was maceration>USE>WBE.

5.4.1.2 Concentration trends analysis

As the main goal of the extractions was identification of compounds present, not as much emphasis was placed on quantification of the extracts. The estimated quantities were only used as supplementary data to assist with identification of these trends. The results can be seen in Addendum A.

5.4.1.2.1 Fresh yellow onion plant material

The data for fresh yellow onion plant material can be seen in Addendum A, table A.1. When the extraction processes were completed for the extracts from whole samples, the general trend showed that the concentration of C decreased, K stayed consistently steady and Q increased over the extraction time periods. This was not dependent on the extraction method (maceration, WBE and USE) being used, with the only outlier being that of Q concentration in WBE that decreased over the extraction period.

When the plant material was in the cut form, it could be seen that the quantities extracted from these samples were overall less than the quantities of the flavonoids released from the whole samples, implying that finer processing causes the flavonoid compounds to break down. No general trend for the specific compounds could be seen, except for the concentration of Q that increased over the extraction time.

With blended plant material, an interesting trend found was that the initial concentration of Q was higher when compared to the initial concentration of both whole and cut extract samples. This suggests that a larger quantity of Q is released after the plant material became damaged or bruised. The general trend seen for C was that the concentration extracted decreased over the extraction time. The concentration of K remained either steady or increased slightly. The concentration of Q showed a general decrease over the extraction period, except during USE.

USE shows a general trend where the concentrations of the compounds increased up to the second extract of the extraction process. Generally, this second extract showed a peak concentration, after which a decrease in extract concentration was seen in the quantities of the final extracts. This leads to the suggestion being made that extraction was either completed, or that the compounds started showing degradation.

Overall, whole samples delivered the best results, with maceration showing the best extraction results. This is followed by USE; and only then WBE. Taking all this into consideration, extracts that performed the best for C were whole samples being macerated. This is also true for K. For Q, the best results were shown by blended onions undergoing USE

5.4.1.2.2 Fresh red onion plant material

The data for fresh red onion plant material can be seen in Addendum A, table A.2.

The general trend seen for whole red onion plant material extracts as time progressed was a decrease in concentration. For USE however, the concentrations of the compounds increased to a peak concentration following the second extract, after which a decrease in concentration is evident as seen in the third extract sample. This decreased concentration was not generally lower than the initial extract sample concentration, but did show a lower concentration than the second extract sample. Another variation seen was that, with maceration, the concentration of C increased as extraction time progressed. With WBE, K showed an increase in concentration over the extraction period.

With cut samples, the general trend identified, looking at maceration and WBE, is that the concentrations for C and K seemed to remain steady over the extraction time period. The concentration of Q seemed to increase, suggesting that finer processing of the plant material by bruising causes Q to be more easily released from the plant material. The trend seen with USE differed from the maceration and WBE processes, as the concentration of the compounds seemed to decrease over the extraction period. K is identified as an outlier in this case, as the trend seen is similar to that for USE, where the second extract sample shows a peak concentration, followed by the decrease seen in the third extract sample. With USE, the concentration of Q decreased in comparison to the trend seen for WBE and maceration.

With the blended samples, no general trend could be identified for the extraction of the samples. It was seen that the concentrations for blended samples were overall lower than those of whole and cut samples undergoing maceration. With WBE and USE, this statement is reversed. Overall with WBE, best extraction values for red onions were obtained from the blended samples.

For the red onions, maceration showed the best results.

5.4.1.2.3 Fresh broccoli plant material

The data for fresh broccoli plant material can be seen in Addendum A, table A.3.

One of the overall trends seen with fresh broccoli, irrespective of extraction method or plant preparation method used, was that the final extract concentration showed a decrease from the second extract sample. In some cases, the second extract sample either followed a trend where the extract concentration remained consistent with that of the first extract concentration; or showed a trend where the concentration obtained from the second extract was a peak concentration value, that followed with a decrease in concentration as seen in the concentration of the third extract sample. An extraction method outlier to this general trend identified in fresh broccoli plant material samples was for the compound Q when the process of maceration was used. Using this extraction method, the concentration of Q showed a general increase. Another outlier identified was for the concentration of K. The concentration of K seemed to stay consistent between the three extracts for whole samples undergoing maceration.

The efficacy of the extraction process may be ranked as follows: USE, maceration and then WBE. The WBEs were not very effective, unless extraction was stopped after the first 30 min. With the blended samples, only K was present after two hours. With the whole samples, a rise in

concentration could be seen for all three compounds. It is still concluded that this was not an effective method for extraction.

Considering the USE, all the whole samples initial concentrations started high, before decreasing after the first hour and then again after the second hour. The only compound that differed is, C which showed an increase in concentration after the second hour. All concentrations were above 1.748 µg/ml, showing that this is an effective method. The blended samples showed an increase in extract concentration after the second hour, before it started declining. It is postulated that the extraction process has been completed, or breakdown could have started occurring at this point. The concentration of Q showed a steep reduction from the initial extract, ending at a low concentration of 0.732 8 µg/ml after the first hour's extraction was completed. This concentration value increased twofold, as could be seen in the extract taken after the second hour. This showed that extracts from whole samples proved to be better.

5.4.1.2.4 Fresh florets plant material

The data for the fresh florets plant material can be seen in Addendum A, table A.4.

Overall, it was harder to identify specific trends after extraction procedures for the florets plant material was completed. What was seen, in comparison to the other plant materials results, was that florets had a greater tendency to be devoid of the analytes. This showed that the extraction process has either stopped, or that the extraction process is slower to start. This could be as the florets plant material has a larger surface area exposed to the extraction solution, in comparison to the other three plant materials used. It is interesting to note that when the plant material was blended, either the initial extract showed improved concentration in comparison to the whole plant material; or the concentrations of the compounds in the extracts improved over the extraction time period, showing a general increase in concentration. This helped in identifying the general trend of a constant increase in concentration over the full extraction period, or a trend where a peak concentration value is seen after the second extract is taken, which was followed by a decline in concentration as seen in the third extract. Highest concentrations obtained from these extractions were for Q.

Overall, WBE results were better than macerated results, showing that force applied during WBE improves extraction over using a constant irritation (as in the case of maceration) replacing saturated solvent with unsaturated solvent. Among the three extraction methods, USE provided the best results where a constant increase in concentration is seen for the first hour, after which

a decline in concentration (which is higher than the initial concentration) is seen. USE proved to be the better extraction method out the three extraction methods being compared.

5.4.1.3 Increase/decrease analysis

In table 5.4 the increase or decrease in extraction is shown when the concentrations in table A.1, A.2, A.3 and A.4 are observed over time:

Table 5.4: The increase/decrease in extraction concentrations (µg/ml) of fresh samples over the full time period of fresh plant materials.

Increase/Decrease										
		Extractions (Fresh)								
		Yellow onions								
		Maceration			Water bath			Ultrasonication		
		1 hour	4 hour	8 hour	30 min	60 min	120 min	30 min	60 min	120 min
Whole	Chrysin	67.421	↓	↓	1.286	↓	↑	0.410	↑	0.000
	Kaempferol	5.756	↓	↑	0.918	↑	↑	1.304	↑	↑
	Quercetin	4.915	↑	↑	13.949	↓	↑	39.170	↓	↑
Cut	Chrysin	1.484	↓	↓	0.325	↑	↓	0.256	↑	↓
	Kaempferol	2.433	↓	↑	0.804	↑	↑	1.366	↑	↓
	Quercetin	4.400	↑	↑	1.332	↓	↑	10.339	↑	↓
Blended	Chrysin	0.427	↓	↓	22.448	↓	↓	0.508	↓	↑
	Kaempferol	0.861	↓	↑	3.635	↓	↓	1.170	↓	↑
	Quercetin	9.838	↓	↓	147.161	↓	↓	16.822	↑	↓
		Red onions								
		Maceration			Water bath			Ultrasonication		
		1 hour	4 hour	8 hour	30 min	60 min	120 min	30 min	60 min	120 min
Whole	Chrysin	11.504	↓	↑	0.452	↓	↓	0.544	↑	↓
	Kaempferol	9.907	↓	↑	0.506	↑	↑	0.842	↑	↓
	Quercetin	79.930	↓	↑	1.364	↑	↓	11.662	↑	↓
Cut	Chrysin	5.204	↓	↑	0.242	0.000	↑	0.373	↓	↑
	Kaempferol	2.037	↓	↑	1.181	0.000	↑	0.814	↑	↓

	Quercetin	2.596	↓	↑	0.635	↑	↑	7.428	↓	↑
	Chrysin	1.314	↓	↑	23.642	↓	↓	0.236	↓	↑
	Kaempferol	1.210	↓	↑	8.126	↓	↓	1.032	↑	↓
Blended	Quercetin	3.131	↓	↑	9.631	↓	↑	9.381	↓	↓
		Broccoli								
		Maceration			**Water bath**			**Ultrasonication**		
		1 hour	4 hour	8 hour	30 min	60 min	120 min	30 min	60 min	120 min
	Chrysin	6.004	0.000	1.352	0.000	↑	0.000	18.965	↓	↑
	Kaempferol	1.820	↓	↑	0.527	↑	0.000	3.609	↓	↑
Whole	Quercetin	2.528	↑	↑	1.452	↑	0.000	30.846	↓	↓
	Chrysin	0.000	↑	↓	0.340	0.000	0.000	0.252	↑	0.000
	Kaempferol	0.497	↑	↓	0.360	↑	↓	0.096	↑	↓
Blended	Quercetin	0.152	↑	↑	18.954	0.000	0.000	11.658	↓	↑
		Florets								
		Maceration			**Water bath**			**Ultrasonication**		
		1 hour	4 hour	8 hour	30 min	60 min	120 min	30 min	60 min	120 min
	Chrysin	0.361	↓	↓	0.496	↓	↑	1.215	↑	0.000
	Kaempferol	0.660	↓	↑	0.586	↓	↑	0.855	↑	↓
Whole	Quercetin	8.920	↓	↓	26.369	↓	↓	0.000	0.000	↑
	Chrysin	0.040	↑	↓	5.252	↓	↑	2.170	0.000	0.000
	Kaempferol	0.000	↑	↓	4.882	↓	↓	2.043	↑	↑
Blended	Quercetin	1.459	↑	↑	21.791	0.000	0.000	36.833	↑	↓

* ↑ - increase in concentration; ↓ - decrease in concentration.

The above table shows the change in concentration as it increased and decreased over the extraction period, irrespective of the specific concentration. Only the starting concentration was given and when the concentration was 0 µg/ml. Considering this, if the second extraction sample and third extraction sample increased in concentration, this is considered to be a more effective extraction, especially if this were the case for all three compounds. It is still advised to consider taking into account the concentration analysis, rather than just an increase or decrease analysis. The order in which extractions should then be considered better is ↑↑>↑↓>↑0>↓↑>↓↓>↓0>0↑>00, with the first symbol representing the second extracted sample, and the second symbol the third sample as can be seen in table 5.4.

Thus, for yellow onions, whole and cut samples undergoing WBE proved to be the better extraction method. For red onions, the increase and decrease was more similar for different samples, leading to the conclusion that maceration could be the best as it proved to be more consistent. Broccoli extractions showed that blended samples undergoing maceration or USE proved to be better methods for extraction. For florets, blended samples undergoing maceration proved better.

5.4.2 Frozen plant samples

5.4.2.1 Presence analysis

Table 5.5 shows the presence of C, K and Q in frozen extract results obtained after analysis:

Table 5.5: The presence of compounds chrysin, kaempferol and quercetin in frozen analysed plant samples.

Presence									
	Extractions (frozen)								
	Yellow Onion								
	Maceration			Water bath			Ultrasonication		
	1 hour	4 hour	8 hour	30 min	60 min	120 min	30 min	60 min	120 min
Whole	C;K;Q	C;K;Q	C;K;Q	C;K;Q	C;Q	C;K;Q	C;Q	C;Q	C;K;Q
Cut	C;Q	C;Q	C;Q	C;K;Q	C;Q	C;Q	C	C;Q	C;Q
Blended	C;Q	C;Q	C;Q	C;Q	C;Q	C	C;K;Q	C;K;Q	C;K;Q
	Red Onion								
	Maceration			Water bath			Ultrasonication		

	1 hour	4 hour	8 hour	30 min	60 min	120 min	30 min	60 min	120 min
Whole	C;Q	C;K;Q	C;Q	C;Q	C;Q	C;K;Q	Q	K;Q	K;Q
Cut	K;Q	C;K;Q	K;Q	C;K;Q	C;K;Q	C;K;Q	C;K;Q	C;K;Q	C;K;Q
Blended	K;Q	K;Q	K;Q	C;K;Q	C;K;Q	C;K;Q	C;K;Q	C;K;Q	C;K;Q
	Broccoli								
	Maceration			**Water bath**			**Ultrasonication**		
	1 hour	4 hour	8 hour	30 min	60 min	120 min	30 min	60 min	120 min
Whole	C;K;Q	C;K	C;K;Q	C;K;Q	C;K;Q	C;K;Q	C;K;Q	C;K;Q	C;K;Q
Blended	C;K;Q	C;K;Q	C;Q	C;K;Q	C;K;Q	C;K	C;Q	C;K;Q	C;K;Q
	Florets								
	Maceration			**Water bath**			**Ultrasonication**		
	1 hour	4 hour	8 hour	30 min	60 min	120 min	30 min	60 min	120 min
Whole	C;K;Q	C;K;Q	C;K;Q	C;K;Q	C;K;Q	C;K;Q	C;K;Q	C;K;Q	C;K;Q
Blended	C;K;Q	C;K;Q	C;K;Q	C;K;Q	C;K;Q	C;Q	C;K;Q	C;K;Q	C;K;Q

In this table, quercetin is shown by Q, kaempferol by K and chrysin by C.

It should be mentioned that in comparison with fresh samples, the freezing process had an influence on the presence of K in the extracts obtained from yellow onion plant materials. This was the same for C in red onions. The extract results for frozen broccoli seemed to be consistent with the results obtained for the extract results from fresh samples. Comparing frozen and fresh florets, it shows that the freezing process could have possibly influenced the amount of compound present, as Q and C were not as evident in the case of USE. This shows that the freezing process influenced the extracted quantities more, as a lower concentration of the compounds was available for extraction, possibly because of destruction of the compounds caused by the freezing process before extraction was started.

5.4.2.2 Concentration trend analysis

5.4.2.2.1 Frozen yellow onion plant material

The data for the frozen yellow onion plant material can be seen in Addendum A, table A.5.

It can be seen from this data that the freezing process affected the quantity of compounds available for extraction during the different extraction by either breaking the compounds down, or preventing its release during extraction. This is especially seen for the concentration of K in the

different plant preparation procedures followed, as in some cases, K was completely absent in the extracted samples. This tendency was evident as the plant preparation procedure increased (blended>cut>whole), meaning this increase could be seen more clearly as the surface area exposed to the extraction solution enlarged. This could possibly have caused destruction of the compound, be it mechanical (through crushing) or loss by freezing. It also showed that the concentrations of the different compounds seemed lower and suggested that frozen plants do not provide the amount of flavonoids in the diet when compared to the extract results from fresh samples.

Overall, the freezing process had the effect that subsequent extracts from the extraction procedure was either more constant in concentration (with a very slight increase or decrease variation), or decreased over the extraction time. Another interesting note to be made is that the concentrations seemed to be lower when compared to extracts results from fresh plant material. This could be due to the effect of freezing, or the added water content, causing the actual plant material weighed being less, as the water content increased and the plant material quantity decreased.

Whole samples seemed to have a general trend in which a decrease in concentration could be seen. The only method which did not show this trend consistently, was USE. This could be due to the cavitation action that causes heating, leading to defrosting of the plant material. This could in effect lead to a greater release of the chemical compounds from the plants, explaining the slight increase in concentration that can be seen.

Cut samples showed a trend in which the concentrations of the compounds being extracted remained more constant over the extraction period. With these extract samples, the concentration of Q did not increase as extraction continued over the extraction period.

Blended samples showed a trend where the concentrations of C and K were lower than that of whole and cut samples. These compounds extract concentrations remained either constant over the extraction period, or showed a decrease in concentration in subsequent extractions. The general trend seen for Q, in all cases, was that the concentration of this compound decreased as extraction time progressed, when looking at the results from subsequent extract samples.

5.4.2.2.2 Frozen red onion plant material

With the red onion plant material, it is interesting to note that the freezing process also slightly affected the concentration available for extraction of C, and not only that of K (as seen in yellow

onion extract results). One of the more general trends seen with red onion plant material is that the extract concentrations followed a trend during the extraction procedure where the concentration of the compounds increased (showing a peak concentration after the second extract was taken), followed by a decrease in concentration as seen in the final extract samples. This suggests that red onions were not affected by the freezing procedure to the same degree that yellow onion plant material was. This led to a trend being identified where more of the flavonoid compounds were released as the plant material became defrosted, leading to the peak concentration seen in the second extract. This was followed by a decline in extract concentration as the extraction procedure had completed during the extraction time. In some cases, finer processing of the plant material also caused a greater release of the flavonoid compounds. In some cases, the complete opposite is true. Overall, blended samples provided excellent results, especially in the case of USE. For the frozen red onions, better overall extraction results were obtained by whole samples undergoing USE for Q; and maceration of whole samples for K and C.

5.4.2.2.3 Frozen broccoli plant material

The frozen broccoli plant material proved to be more tolerant to the freezing process than either the yellow or red onions, as the absence of the flavonoid compounds was not as prominent in the broccoli extract samples. At different points in the extraction procedure (either being the initial or final extract), a specific compound does seem to be absent from the extract sample in the case of K and Q. If it was the initial extract, it could suggest that release of the flavonoid compound into the extract solution took longer, or penetration of the extract solution into the plant material progressed at a slower tempo, being dependent on the defrosting tempo. If it was the final extract, it could suggest that the extraction process had concluded. No specific general trend could be identified, except for the concentration being higher in the order of Q>K>C. Interestingly enough, WBE provided better results than both maceration and USE, in the order of whole samples more than blended samples.

5.4.2.2.4 Frozen florets plant material

The data for frozen florets plant material can be seen in Addendum A, table A.8.

Some of the extract samples used with the florets extraction proved to have higher concentrations than those observed after completion of fresh florets extractions. It is interesting that the florets

frozen plant material concentrations of all compounds were higher than those seen in the results obtained from frozen broccoli. All compounds were present in all extraction procedures extract samples, except for Q in the blended florets undergoing WBE final extract sample. This shows that the extraction procedure had concluded, as the trend followed is where a peak concentration is seen in the second extract, before the decrease to a 0 µg/ml value as seen in the final extract.

No specific trends were identified, as some extracts showed a general increase or decrease in concentration, while others showed a general decrease that stayed constant in concentration through the extraction procedure. In some extraction procedures, these two trends were mixed, leading to the final trend identified, being an increase in extract concentration that peaked as seen in the second extract sample, followed by a decrease in concentration seen in the final extract sample. As broccoli has shown to be more tolerant to freezing than onion plant material (affecting availability of compounds for extraction by either destruction or slower release), it seems the florets part (the dark green flowery part without the stems) of the broccoli (florets and stems included) to be even more resistant to effects from the freezing process, which could possibly cause the higher flavonoid content of extract samples. Whole plant material does seem to constantly deliver better extraction concentrations, in the order of maceration>USE>WBE.

Overall the results for fresh plant material proved to be better than that of frozen plant material, suggesting that edible fresh plants are still healthier for ingestion than frozen plant material.

5.4.2.3 Increase/decrease analysis

In table 5.6 the increase or decrease in extraction is shown when the concentrations in table A.5, A.6, A.7 and A.8 are observed over time:

Table 5.6: **The increase/decrease in extraction concentrations (µg/ml) of frozen samples over the full time period for frozen plant materials.**

Increase/Decrease										
		Extractions (Frozen)								
		Yellow Onion								
		Maceration			Water bath			Ultrasonication		
		1 hour	4 hour	8 hour	30 min	60 min	120 min	30 min	60 min	120 min
Whole	Chrysin	6.256	↓	↓	1.107	↓	↑	0.188	↓	↑
	Kaempferol	5.344	↓	↓	2.328	0.000	↑	0.000	0.000	↑
	Quercetin	16.052	↓	↓	5.953	↓	↑	7.965	↓	↓
Cut	Chrysin	0.333	↑	↓	0.073	↑	↑	0.216	↓	↑
	Kaempferol	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Quercetin	2.315	↑	↓	0.000	↑	↑	0.000	↑	↑
Blended	Chrysin	0.290	↓	↓	0.401	↓	↓	2.339	↑	↓
	Kaempferol	0.000	0.000	0.000	0.000	0.000	0.000	2.818	↑	↓
	Quercetin	3.111	↓	↓	10.352	0.000	0.000	10.827	↓	↓
		Red Onion								
		Maceration			Water bath			Ultrasonication		
		1 hour	4 hour	8 hour	30 min	60 min	120 min	30 min	60 min	120 min
Whole	Chrysin	3.015	↑	↓	0.199	↓	↑	0.000	0.000	0.000
	Kaempferol	0.000	2.526	0.000	0.000	0.000	↑	0.000	↑	↑
	Quercetin	9.449	↑	↓	7.464	↓	↑	17.795	↑	↑
Cut	Chrysin	0.000	↑	0.000	0.127	↑	↑	0.452	↑	↓
	Kaempferol	1.966	↓	2.602	0.940	↑	↓	0.587	↑	↓
	Quercetin	1.335	↑	↓	1.235	↑	↓	3.643	↑	↑

Blended	Chrysin	0.000	0.000	0.000	0.449	↓	0.445	0.295	↑	↓
	Kaempferol	0.811	↑	↓	2.456	↓	↑	1.491	↑	↓
	Quercetin	8.381	↑	↑	4.450	↓	↑	1.753	↑	↓
		Broccoli								
		Maceration			**Water bath**			**Ultrasonication**		
		1 hour	4 hour	8 hour	30 min	60 min	120 min	30 min	60 min	120 min
Whole	Chrysin	0.393	↑	↑	25.528	↓	↓	0.603	↓	↑
	Kaempferol	1.522	↑	↓	5.799	↓	↑	2.140	↓	↑
	Quercetin	1.374	0.000	↑	4.590	↓	↑	2.288	↑	↓
Blended	Chrysin	0.499	↓	↓	0.548	↑	↓	0.093	↓	↑
	Kaempferol	1.226	↑	0.000	2.859	↓	↑	0.000	↑	↓
	Quercetin	1.269	↓	↑	1.479	↑	0.000	3.838	↓	↑
		Florets								
		Maceration			**Water bath**			**Ultrasonication**		
		1 hour	4 hour	8 hour	30 min	60 min	120 min	30 min	60 min	120 min
Whole	Chrysin	2.279	↑	↓	0.187	↑	↑	5.294	↓	↓
	Kaempferol	15.822	↓	↓	1.748	↓	↑	2.549	↑	↓
	Quercetin	49.052	↑	↓	8.261	0.000	↑	11.615	↑	↓
Blended	Chrysin	2.201	↓	↑	0.815	↑	↓	0.800	↓	↑
	Kaempferol	33.463	↓	↑	0.924	↓	↓	8.331	↓	↓
	Quercetin	23.502	↓	↑	1.749	↑	0.000	11.292	↓	↓

* ↑ - increase in concentration; ↓ - decrease in concentration.

As mentioned with fresh plant material, if concentrations are not taken into account, the best extractions for C in the case of frozen plant material is cut samples undergoing WBE for yellow and red onions, whole samples undergoing maceration for broccoli and whole samples undergoing WBE for florets. For K, blended USE proved to give better results for yellow onions and in the case of red onions, this seemed to be whole samples undergoing USE.

With the broccoli, there was no extraction procedure that showed a constant increase over the extraction period, giving more than one option for the most favoured extraction method, being maceration or USE of whole plant material; and USE for blended plant material. But as is the case with onion plant material, it is advised to use USE, minimising the extraction time, solvent volume and plant material necessary for extraction. USE with whole florets plant material also proved to be better. When looking at specific compounds, considering Q, better extract results were obtained for cut yellow onion plant material undergoing WBE or USE. In the case of red onion plant material, the options considered as better extraction methods and plant processing procedures, it is advised to use whole or cut plant material undergoing USE; or blended plant material undergoing maceration. Since USE is a suitable extraction method in both instances, this may be considered to be the better extraction method for frozen plant material. Broccoli showed better results for whole plant material undergoing USE, while for florets, either maceration or USE of whole plant material yielded better results.

5.4.3 Microwave assisted extractions of plant materials

5.4.3.1 Presence analysis

Table 5.7 shows the presence of C, K and Q in frozen extracts that have been made after analysis:

Table 5.7: The presence of compounds chrysin, kaempferol and quercetin in analysed plant extract samples using MAE.

Presence						
	Extractions (MAE)					
	Yellow onion					
	Mi 60			Mi 80		
	10 s	30 s	60 s	10 s	30 s	60 s
Whole	C;K;Q	C;K;Q	C;K;Q	C;K;Q	C;K;Q	C;K;Q
Cut	C;K;Q	C;K;Q	C;K;Q	C;K;Q	C;K;Q	C;K;Q
Blended	K;Q	K;Q	C;K;Q	C;K;Q	C;K;Q	K;Q

	Red onion					
	Mi 60			Mi 80		
	10 s	30 s	60 s	10 s	30 s	60 s
Whole	C;Q	C;K;Q	C;K;Q	C;K;Q	K;Q	C;K;Q
Cut	K;Q	C;K;Q	C;K;Q	C;K;Q	C;K;Q	K;Q
Blended	Q	C;Q	C;Q	C;K;Q	C;K;Q	C;K;Q
	Broccoli					
	Mi 60			**Mi 80**		
	10 s	30 s	60 s	10 s	30 s	60 s
Whole	C;K;Q	C;K;Q	C;K;Q		C;K;Q	C;K;Q
Blended	C;K;Q	C;K	C;K;Q	C;K;Q	C;K;Q	C;K;Q
	Florets					
	Mi 60			**Mi 80**		
	10 s	30 s	60 s	10 s	30 s	60 s
Whole	C;K;Q	C;K;Q	C;K;Q		C;K;Q	C;K;Q
Blended	C;K;Q	C;K;Q	C;K;Q	C;K;Q	C;K;Q	C;K;Q

In this table, quercetin is shown by Q, kaempferol by K and chrysin by C.

As can be seen in table 5.7, in MAE extracts, most of the compounds were present. When yellow onions were blended, C was often absent from the extract. For red onions, C and K were often absent from the extracts. Interestingly, no compounds were present when broccoli and florets were microwaved at 80% power after the first 10 s, but did show in the 30 s and 60 s extracts. Overall, C, K and Q was present.

5.4.3.2 Concentration trend analysis

5.4.3.2.1 Yellow onion plant material MAE

The concentration data for yellow onion plant material that has been subjected to MAE is given in Addendum A, table A.9.

MAE will be discussed in an order where all extract samples undergoing MAE at 60% power is first compared, followed by extracts undergoing MAE at 80%, and then comparing the 60% and 80% results with each other. Overall, the results obtained for the extract concentrations of these samples undergoing MAE tended to be higher in comparison to results obtained for fresh and frozen plant samples undergoing maceration, WBE and USE. The overall trend that could be constantly seen over the full extraction period, is a rise in extract concentration leading up to the

second extract taken at 30 s (showing a peak concentration), followed by a decrease in concentration (as seen in the 60 s extract concentrations) as degradation of the compounds occurred.

Comparing extract results obtained from MAE at 60% power with results obtained from frozen or fresh whole plant material undergoing extraction using the process of maceration, it is interesting to mention that the highest concentrations of compounds in the initial extract from MAE was in the order of K>C>Q. This pattern completely differed from the one as seen in samples obtained from maceration. This pattern remained almost constant for all yellow onion plant material extract results, suggesting that MAE either enhances the extraction of K more than that of Q, or enhances the breakdown process of Q by conversion to C or K (there is no evidence that this was possible). The order in which the compound concentrations in the subsequent extracts (30 s and 60 s extracts) was rearranged in favour of Q (being K>Q>C). With whole samples, the initial trend seen was that the already high compound concentrations obtained from the 10 s extracts increased to a peak concentration as can be seen in the 30 s extract sample, which then started to decrease (possibly because of degradation) as can be seen from results obtained from the 60 s extract.

With cut samples, the order for highest to lowest compound concentrations for the initial extract changed to K>Q>C, when compared to whole samples. This order rearranged for subsequent extracts as can be seen in the 30 s extract sample (Q>K>C) which was seen until the end of the 60 s extraction period. The concentration of C was very low when this extraction was completed, showing that microwaves applied causing heating of the plant matrix and thus extract solution, affected C more than K or Q. The blended samples showed the normal order of Q>K>C, as seen in fresh and frozen plant material results. Interestingly enough, the concentrations of the compounds in the blended samples kept on increasing as MAE continued.

Comparing MAE results at 80% power, the same trends could be seen. The difference being that overall concentrations from extracts were lower, which suggested that the higher energy input caused enhanced degradation. The only difference when comparing Mi 60 to Mi 80 results, was with the results obtained from blended plant material as the concentrations of the compounds in the Mi 80 extract results were higher than those seen in extracts from blended plant material extracts at 60% power. Out of the above, it is thus evident that Mi 60 results tend to be better. Another trend seen was that by finer processing of yellow onion plant material, compound concentrations in the subsequent extracts decreased.

5.4.3.2.2 Red onion plant material MAE

The concentration data for red onion plant material that has been subjected to MAE is given in Addendum A, table A.10.

When comparing results obtained from red onion plant material extractions to those obtained for yellow onion plant material, overall, it was not possible to identify a constant extraction trend. This could possibly mean that red onion plant material was not as heat labile as yellow onion plant material. One of the general trends seen was with the Mi 60%, where a more constant increase in concentration can be seen in the extracts. As soon as the power was raised (Mi 80%), extraction concentrations of the compounds decreased, or reached a peak concentration (as seen in the 30 s extract samples), before a decrease in concentration is seen (possibly suggesting degradation). Comparing the results for the three chemical compounds, it was seen that C is not as heat labile as K or Q. Overall, higher extraction concentrations were reached using this method.

With the red onion MAEs, the general order of extraction concentrations from highest to lowest shifted back to Q>K>C. Overall, the concentrations were lower than the ones obtained from yellow onion plant material extracts.

As seen from table A.10, it may be concluded that using MAE for red onion plant material (by processing the onion into finer pieces), extraction is enhanced by increasing the power for extraction. The opposite seems to be true for Mi 60, as the order in which compound concentrations in the extract samples improved, was whole>cut>whole. Thus, in the case of red onion plant material, better extract results depend on the processing and power used for MAE to determine the best extraction method.

5.4.3.2.3 Broccoli plant material MAE

The concentration data for broccoli plant material that has been subjected to MAE is given in Addendum A, table A.11.

In some cases, the MAE results seemed better than that of fresh and frozen samples. This depends on the compound being examined for which, in some cases, the previous two methods had better results. There were two prominent trends identified, being either a continuous increase, or a fluctuation where the 30 s extraction showed a decrease in concentration of the compound before an increase is seen again.

Overall, K showed a general increase in all extracts obtained from this extraction method as extraction time progressed. This was not true for the concentrations of C and Q, showing K to be more heat labile. In comparison to the onion plant material samples, the broccoli showed better extraction results when MAE was carried out at the higher power setting (Mi 80). It was seen from the extracts that, for the concentration of Q, the more the plant material had been processed (blended>cut>whole), the better the extraction concentration obtained for the compound was. At the lower power setting (Mi 60), it led to a higher concentration being obtained after the extraction period had been completed.

In conclusion, the Mi 80 results showed greater increases in concentration of the compounds for whole broccoli than those obtained from Mi 60. The only exception was the concentration of C, which as previously mentioned, seemed to be more susceptible to degradation when microwaves were applied.

5.4.3.2.4 Florets plant material MAE

The concentration data for florets plant material that has been subjected to MAE is given in Addendum A, table A.12.

The florets showed more random extraction patterns than the other MAE results. This could be due to the larger surface area, showing greater penetration, or degradation that occurred. Comparing the concentrations of the compounds in the extract samples, in some cases, the concentrations using MAE were higher than those obtained from maceration, WBE and USE methods used as extraction procedures for fresh and frozen plant material. As only some of the results were better, some of the compound concentration results obtained from fresh and frozen plant material was higher than that of MAE. For florets, the better extraction results were thus obtained in the order of MAE>frozen (maceration>USE>WBE)>fresh (maceration>USE>WBE).

The whole samples showed a trend where peak concentrations of K and C were reached after 30 s, before starting to decrease as seen in the 60 s extracts. At Mi 80, this degradation was not seen for K, which rather showed a general increase in compound degradation in the subsequent extracts taken. The concentration of Q increased consistently over the extraction period. Higher concentrations for compounds extracted from florets plant material was achieved using MAE at 60%, rather than 80%, irrespective whether the samples were whole or blended. With the blended samples, the initial extract's compound concentrations were higher than compound concentrations obtained from whole plant material extract samples. This showed a general decrease in compound concentration when MAE was applied at 60% power, while an increase in

compound concentration was evident when MAE was applied at 80%, the only exception being for the concentration of C. Overall the best results were achieved by microwave assisted extraction applied at 60% power.

Overall, the compound concentrations obtained with MAE proved to be higher than compound concentrations obtained from other extraction methods.

5.4.3.3 Increase/decrease analysis

In table 5.8 the increase or decrease in extraction is shown when the concentrations in table A.9, A.10, A.11 and A.12 are observed over time:

Table 5.8: The increase/decrease in compound concentrations (µg/ml) obtained from extracts undergoing MAE over the full extraction time period for plant materials.

Increase/Decrease								
		Extractions (MAE)					
		Yellow Onion					
		Mi 60			Mi 80		
		10 s	30 s	60 s	10 s	30 s	60 s
Whole	Chrysin	15.473	↑	↓	10.166	↑	↓
	Kaempferol	19.931	↑	↓	12.877	↑	↓
	Quercetin	14.832	↑	↓	9.995	↑	↓
Cut	Chrysin	6.474	↑	↓	4.637	↑	↓
	Kaempferol	10.809	↑	↓	15.852	↑	↓
	Quercetin	8.654	↑	↓	0.601	↑	↓
Blended	Chrysin	0.000	0.000	↑	1.043	↑	0.000
	Kaempferol	0.228	↑	↑	1.179	↑	↓
	Quercetin	1.826	↑	↑	7.060	↑	↓
		Red Onion					
		Mi 60			Mi 80		
		10 s	30 s	60 s	10 s	30 s	60 s
Whole	Chrysin	2.311	↑	↑	2.227	0.000	↑
	Kaempferol	0.000	↑	↑	5.594	↓	↑
	Quercetin	22.935	↑	↑	10.317	↓	↑
Cut	Chrysin	0.000	↑	↓	5.263	↓	0.000
	Kaempferol	0.962	↑	↑	8.820	↓	↓

	Quercetin	14.583	↑	↑	12.766	↑	↓
Blended	Chrysin	0.000	↑	↑	7.013	↑	↓
	Kaempferol	0.000	0.000	0.000	11.050	↓	↓
	Quercetin	12.235	↓	↑	23.098	↑	↑
		Broccoli					
		Mi 60			**Mi 80**		
		10 s	30 s	60 s	10 s	30 s	60 s
Whole	Chrysin	7.321	↓	↑	0.000	↑	↑
	Kaempferol	2.558	↑	↑	0.000	↑	↑
	Quercetin	6.050	↓	↑	0.000	↑	↑
Blended	Chrysin	0.628	↓	↑	0.703	↑	↓
	Kaempferol	3.059	↓	↑	2.949	↑	↑
	Quercetin	2.869	0.000	↑	26.566	↓	↓
		Florets					
		Mi 60			**Mi 80**		
		10 s	30 s	60 s	10 s	30 s	60 s
Whole	Chrysin	1.463	↑	↓	0.000	↑	↓
	Kaempferol	2.158	↑	↓	0.000	↑	↑
	Quercetin	3.531	↑	↑	0.000	↑	↑
Blended	Chrysin	1.744	↓	↑	1.007	↓	↑
	Kaempferol	23.194	↓	↓	7.757	↑	↑
	Quercetin	30.469	↓	↓	12.151	↑	↑

* ↑ - increase in concentration; ↓ - decrease in concentration.

The above table compares the increase and decrease of concentrations of C, K and Q according to the plant material and MAE power used. For yellow onions, the blended samples undergoing MAE at 60% provided the best results for K and Q. For C, all methods seem to have the same efficiency. For red onion plant material, the overall best results were obtained from MAE of whole samples. The concentrations of K and Q in the cut samples showed a constant increase. For the blended samples, this could also be seen for C. All of these results were obtained from MAE at 60%. From the Mi 80 results, the only extraction that showed the same trend was for Q in blended samples. Thus for onions, the best results were obtained with MAE at Mi 60.

The broccoli extracts showed, overall, that whole samples microwaved at 80% provided the best results. The blended samples provided the same constant increase in concentration for K. Microwaving at 60%, K was the only compound from the extractions that showed this same trend for whole samples. For florets, C had the best results for MAE at 60% or 80% for whole samples. K and Q showed better results for whole and blended samples at 80% power. Whole florets plant

material undergoing MAE at 60% power provided efficient results when looking at the compounds concentrations from the extracts. The best results for broccoli and florets could thus be obtained by Mi 80, rather than Mi 60 as in the case of the onions. This shows that increasing power had a better effect on the extraction efficacy of broccoli than on onions.

5.4.4 Cooked plant samples

5.4.4.1 Presence analysis

Table 5.9 shows the presence of C, K and Q in extracts obtained from cooked plant material samples after analysis:

Table 5.9: The presence of compounds chrysin, kaempferol and quercetin in cooked analysed plant samples.

Presence						
	Extractions (Cooked)					
	Yellow Onion			Red Onion		
	Maceration					
	30 min	60 min	120 min	30 min	60 min	120 min
H2O	C;K	Q	Q	C;Q	C;Q	C;Q
Sample	C;K;Q	C;Q	C;Q	C;K;Q	C;K;Q	C;Q
	Broccoli			Florets		
	Maceration					
	30 min	60 min	120 min	30 min	60 min	120 min
H20	Q	Q	Q	Q	Q	Q
Sample	Q	Q	Q	C;Q	Q	Q

In this table, quercetin is shown by Q, kaempferol by K and chrysin by C.

From this table it is evident that the cooking procedure using water affects the concentrations of C, K and Q present in different plant materials available for extraction. Some of the analytes are lost during cooking, partitioning to the water used during the cooking procedure and are thus rather present in the water than in the plant material. During this procedure, the compounds were either present in the water or completely destroyed/absent in the plant material. With onions, C and Q are constantly present, while red onion plant material showed that after a long cooking

period, K was absent. Considering broccoli and florets, it could be seen that the cooking procedure completed the extraction procedure, or almost completely degraded C and K, though C was still present after a few minutes in the florets material after cooking. Q showed a loss from the plant material and was present in the water.

5.4.4.2 Concentration trend analysis

Table 5.10 shows the calculated concentrations using AUC compared to the AUC of the standards. The AUC of M was compared to a 10 µg/ml standard with which the concentration was calculated. The concentrations of the Q, K and C could thus be calculated by comparing the AUC of the samples to the AUC of the M. It has to be taken into consideration that the concentration difference of the standard samples used for analysis were 10 µg/ml, instead of 1 mg/ml, leading to the immense difference that can be seen in the concentrations obtained:

Table 5.10: Calculated concentrations of chrysin, kaempferol and quercetin in the extracts from cooked plant material and the water used as time progressed for the cooking procedure.

Sample concentrations:				
Sample	**Compound**	**[µg/ml] sample 1**	**[µg/ml] sample 2**	**[µg/ml] sample 3**
Yellow onion				
H_2O	**Chrysin**	0.006	0.000	0.000
	Kaempferol	0.004	0.000	0.000
	Quercetin	0.000	0.116	0.021
Maceration	**Chrysin**	0.049	0.004	0.002
	Kaempferol	0.005	0.000	0.000
	Quercetin	0.075	0.072	0.085
Red onion				
H_2O	**Chrysin**	0.002	0.002	0.001
	Kaempferol	0.000	0.000	0.000
	Quercetin	0.030	0.036	0.047
Maceration	**Chrysin**	0.003	0.002	0.001
	Kaempferol	0.003	0.003	0.000
	Quercetin	0.108	0.137	0.085
Broccoli				
H_2O	**Chrysin**	0.000	0.000	0.000
	Kaempferol	0.000	0.000	0.131

	Quercetin	0.039	5.174	5.953
Maceration	**Chrysin**	0.013	0.000	0.000
	Kaempferol	0.030	0.000	0.000
	Quercetin	2.638	1.875	3.733
Florets				
H_2O	**Chrysin**	0.000	0.000	0.000
	Kaempferol	0.000	0.000	0.000
	Quercetin	2.194	3.881	4.421
Maceration	**Chrysin**	0.003	0.000	0.000
	Kaempferol	0.000	0.000	0.000
	Quercetin	2.228	4.921	1.685

Considering the results for yellow onions, in the first extract, C, K and Q are present in the order of Q>C>K. The concentrations for C and K in the water was low at this point and absent in subsequent extracts showing that extraction has been completed, or compound degradation had taken place. The concentration of C in the initial plant material extract was low with a constant reduction as the cooking period continued. K was only present in the initial extract sample. The concentration of Q in the onion stayed relatively constant, showing that Q can still be obtained in the diet when the yellow onions are cooked, albeit in a very low concentration.

Red onions had all three compounds more constantly present in the cooked plant material as shown by subsequent extract results. The concentration of C and K started very low, showing a decrease in concentration over the full extraction period. The concentration of Q was the highest, showing a peak concentration that was reached in the second extract, only decreasing after this as seen in the final extract. The concentration of C was a bit lower in the water than in the plant material and showed a decrease over the cooking period. K was absent in the water samples taken at the same time as the extractions. As time progressed, even though the Q concentration was quite low, it increased steadily as cooking continued.

For the cooked broccoli plant material, all compounds were present in the initial extract taken. After this, only Q was present, showing a decrease in concentration in the extract of the second sample, and then an increase again. This also suggests that Q is more tolerant to heat than the other compounds. In the water, only Q was present, showing a huge increase in concentration as the quantity in the water was greater than the quantity seen in the plant extracts. K was only present in the last water sample at a low concentration.

With the florets, only a very low concentration of C and Q was present in the initial extract sample. Only Q was present in the second and third extracts, showing a peak concentration that

decreased over the extraction time. The florets had a higher concentration of Q in comparison to all other cooked plant materials. In the water extractions, only Q was present showing an increase in concentration as the cooking procedure continued. Comparing the final extracts, more Q was in the water sample, than in the plant extract sample.

5.4.4.3 Increase/decrease analysis

In table 5.11 the increase or decrease in extraction is shown when the concentrations in table 5.10 is observed over time:

Table 5.11: The increase/decrease in extraction concentrations (µg/ml) of cooked samples over the full time period for cooked plant materials.

Increase/Decrease							
Extractions (Cooked)							
		Yellow onion			Red onion		
		Maceration			Maceration		
		30 min	60 min	120 min	30 min	60 min	120 min
H_2O	Chrysin	0.006	0.000	0.000	0.002	0.002	↓
	Kaempferol	0.004	0.000	0.000	0.000	0.000	0.000
	Quercetin	0.000	↑	↓	0.030	↑	↑
Sample	Chrysin	0.049	↓	↓	0.003	↓	↓
	Kaempferol	0.005	0.000	0.000	0.003	↓	0.000
	Quercetin	0.0747	↓	↑	0.108	↑	↓
		Broccoli			Florets		
		Maceration			Maceration		
		30 min	60 min	120 min	30 min	60 min	120 min
H_2O	Chrysin	0.000	0.000	0.000	0.000	0.000	0.000
	Kaempferol	0.000	0.000	↑	0.000	0.000	0.000
	Quercetin	0.039	↑	↑	2.194	↑	↑
Sample	Chrysin	0.013	0.000	0.000	0.003	0.000	0.000
	Kaempferol	0.030	0.000	0.000	0.000	0.000	0.000
	Quercetin	2.638	↓	↑	2.228	↑	↓

* ↑ - increase in concentration; ↓ - decrease in concentration.

With the cooked samples, the focus was on determining the effect of the cooking procedure on the flavonoid content of the plant material, rather than determining the most effective extraction method. Only one extraction method was used, and that was maceration after cooking.

For yellow onion plant material, the C extract analysis showed a decrease over the extraction period. When comparing the content of the water sample with the plant extract sample, it was lower and immediately decreased to a 0 µg/ml value. K was present in both the initial plant extract and water sample, decreasing to a 0 µg/ml in subsequent extractions. For Q, the extract showed a decrease in compound concentration, followed by an increase in concentration. Comparing the water samples, Q was absent in the initial sample, followed by an increase as seen in the second sample taken; and then decreasing again as seen in the final sample. This suggests that degradation of the compound occurred, irrespective of more Q entering the water from the plant material.

With the broccoli, only Q is discussed. In the extract, the concentration showed a trend where the compound decreased and then increased again. In the water, this content gradually raised as the Q content moved out of the plant material into the water as can be seen in subsequent samples.

The above table suggests that the only plant with a Q content that is not negligible in the diet, is that obtained from broccoli and florets as these plant materials had a bit higher content than onions that were cooked.

5.4.5 Grilled onion extractions

5.4.5.1 Presence analysis

Table 5.12 shows the presence of C, K and Q compounds in grilled onion (butter and margarine) extracts:

Table 5.12: The presence of chrysin, kaempferol and quercetin compounds in grilled onion plant extracts.

Presence:			
Extractions (Grilled onions)			
	Margarine		
	Maceration		
	30 min	60 min	120 min
Margarine	C;Q	-	-
Yellow Onion	C;K;Q	C;K;Q	C;K;Q
Red Onion	C;K;Q	C;K;Q	C;K;Q
	Butter		

	Maceration		
	30 min	60 min	120 min
Butter	C;K;Q	-	-
Yellow Onion	K;Q	C;K;Q	-
Red Onion	C;K;Q	K;Q	C;K;Q

In this table, quercetin is shown by Q, kaempferol by K and chrysin by C.

For the grilled onions, it was necessary to analyse the margarine and butter for the analytes. As seen above, both C and Q are present in margarine. With butter, C, K and Q were present. This is of importance as the flavonoid content from the cooking medium used could influence the analysis of the content by either adding to the quantities seen, or being the only content present in extracted samples after the grilling procedure.

For the grilled onions, only the maceration extraction method was used. As seen from table 5.12, for red and yellow onion plant material grilled in margarine, all compounds were present. When the yellow onion plant material was grilled in butter, only K and Q was present in the initial extract. All compounds were present in the second extract taken after 60 min. In the final extract taken after 120 min, all compounds were absent from the extract taken. Looking at the extracts taken from red onion plant material, all compounds were present in subsequent extracts, the only exception being C, in the 60 min extract. This signifies the difference between the two types of onions used for the procedure, rather than the grilling method being a problem.

5.4.5.2 Concentration trend analysis

Table 5.13 shows the calculated concentrations using AUC compared to the AUC of the standards. The AUC of M was compared to a 1 µg/ml standard with which the concentration was calculated. The concentrations of the Q, K and C could thus be calculated by comparing the AUC of the samples to the AUC of the M:

Table 5.13: Calculated concentrations in the extracts as time progressed for grilled onion plant material.

Sample concentrations:					
Method	**Sample**	**Compound**	**[µg/ml] sample 1**	**[µg/ml] sample 2**	**[µg/ml] sample 3**
	Margarine	**Chrysin**	18.389		

		Kaempferol			
		Quercetin	4.535		
Maceration	**Yellow Onion**	**Chrysin**	3.159	1.861	0.396
		Kaempferol	2.948	2.230	0.958
		Quercetin	3.124	1.678	1.242
	Red Onion	**Chrysin**	0.489	1.262	0.386
		Kaempferol	1.074	2.582	1.201
		Quercetin	15.777	18.955	25.632
	Botter	**Chrysin**	0.723		
		Kaempferol	0.372		
		Quercetin	1.798		
Maceration	**Yellow Onion**	**Chrysin**	0.000	0.835	0.000
		Kaempferol	2.975	1.184	0.000
		Quercetin	1.159	4.633	0.000
	Red Onion	**Chrysin**	1.515	0.000	10.228
		Kaempferol	0.858	0.924	0.849
		Quercetin	1.943	2.742	2.668

As mentioned in the presence analysis, the margarine contained C and Q. The concentration of C content was higher than that of Q. Considering that these two compounds are present in the margarine, this additional quantity could have caused the onion samples to have higher C and Q values in both cases. The initial compound concentration obtained from grilled yellow onion plant material extracts from highest to lowest was C>Q>K, which could indicate a greater content of C being ingested from margarine than the onion plant material. The C content decreased as extraction continued. Even though the concentration of K was lowest at the beginning of the extraction procedure, it was the highest after the extract taken at 60 min and ended as the second highest compound concentration when the extraction period was completed. It followed the same trend as C by decreasing after each extraction. Q also followed this trend, but had the highest concentration when extraction was completed. The red onions trend with C and Q showed a fluctuating trend with the initial concentration first increasing (as seen from the second extract sample) and then decreasing again (as seen from the final extract sample). For the grilled red onion plant material, compound concentrations obtained from the initial extract were highest to lowest in the order Q>K>C. The concentration of Q increased over the extraction period, showing the highest compound concentrations obtained from margarine grilled plant material samples. Better results were obtained for Q from the red onion plant material, while K and C showed better results obtained from yellow onion plant material.

All three compounds were present in butter, with the highest to lowest compound concentration being in the order of Q>C>K. These concentrations were significantly lower than those seen in margarine. For yellow onion plant material, the concentration of C rose from 0 µg/ml to a low peak concentration (as seen in the second extract), before decreasing back to a 0 µg/ml concentration (in the final extract sample). Initially, the concentration of K was the highest. Over the full extraction period, this compound concentration only showed a decrease. Q started with a lower concentration than that of K and rose to a peak concentration (as seen in the second extract sample), before showing a complete absence in the final sample. The concentrations of the analytes in red onion plant material extracts showed a higher concentration of C which increased over the extraction period. The concentration of K showed a fluctuating pattern, as the K concentration increased (as seen in the second extract sample), followed by a decrease in concentration as seen in the final extract sample. Overall, the concentration values of K remained relatively constant. Q also increased in concentration before showing a very slight reduction in concentration. Thus from the butter extracts, better results were obtained for C from the red onion plant material, while K and Q results proved to be better from the yellow onion plant material.

Overall, better results were obtained from the onions grilled in margarine than the ones from butter. This could be ascribed to the fact that margarine is made from plants, while butter comes from animal fat.

5.4.5.3 Increase/decrease analysis

In table 5.14 the increase or decrease in extraction is shown when the concentrations in table 5.13 is observed over time:

Table 5.14: The increase/decrease in extraction concentration (µg/ml) of grilled onion extraction samples over the full time period.

Increase/Decrease				
Extraction (Grilled onions)				
Margarine				
		Maceration		
		30 min	60 min	120 min
Margarine	Chrysin	18.389		
	Kaempferol			
	Quercetin	4.535		
	Chrysin	3.159	↓	↓

Yellow Onion	Kaempferol	2.948	↓	↓
	Quercetin	3.124	↓	↓
Red Onion	Chrysin	0.489	↑	↓
	Kaempferol	1.074	↑	↓
	Quercetin	15.776	↑	↑
Butter				
		Maceration		
		30 min	60 min	120 min
Butter	Chrysin	0.723		
	Kaempferol	0.372		
	Quercetin	1.797		
Yellow Onion	Chrysin	0.000	↑	0.000
	Kaempferol	2.975	↓	0.000
	Quercetin	1.159	↑	0.000
Red Onion	Chrysin	1.515	0.000	↑
	Kaempferol	0.858	↑	↓
	Quercetin	1.943	↑	↓

* ↑ - increase in concentration; ↓ - decrease in concentration.

Only general trends will be mentioned as the concentration analysis yielded a better description seen from these extractions. The general trend for extract samples obtained from yellow onion plant material grilled in margarine was a decrease in concentration. For red onion plant material, C and K first showed an increase in concentration, followed by a decrease in concentration. The concentration of Q only increased over the extraction period.

Using butter as medium, the yellow onion plant material extracts showed an increase for C and Q, before decreasing to a 0 µg/ml concentration. The concentration of K showed a decreasing trend from the start of the initial extract sample, to the end of the final extract sample. The red onion plant material showed a decrease in concentration as seen in the second sample, before an increase in concentration was shown as evident from the final extract sample. For K and Q, the concentrations first increased as seen in the second extract sample, followed by a decrease in concentration as seen from the final extract.

5.4.6 Tea extractions

5.4.6.1 Presence analysis

Table 5.15 shows the presence of C, K and Q in tea extracts from different teas available in South Africa:

Table 5.15: The presence of compounds chrysin, kaempferol and quercetin in different teas using both boiled HPLC water and methanol.

Presence					
Extractions (Teas)					
	Earl Grey®	Five Roses®	Rooibos®	Kilimanjaro®	Dilmah Ceylon®
H_2O	-	Q;K;C	Q;K;C	Q;K;C	Q;K;C
Me	K;C	C	Q;K;C	C;K	C

In this table, quercetin is shown by Q, kaempferol by K and chrysin by C.

With the tea extracts, boiled water was used and compared to extractions using Me. The Me extractions were not as effective as using only water as extraction solvent. The different teas showed all three tested compounds when boiling water was used, while only rooibos tea showed all three compounds using Me as extraction solvent. Earl grey®- and the Kilimanjaro® tea showed both C and K as present, while the five roses- and Dilmah Ceylon® tea showed only C.

5.4.6.2 Concentration analysis

Table 5.16 shows the calculated concentrations using AUC compared to the AUC of the standards. The AUC of M was compared to a 1 µg/ml standard with which a concentration was calculated. The concentrations of the Q, K and C could thus be calculated by comparing the AUC of the samples to the AUC of the M:

Table 5.16: Estimated concentrations of chrysin, kaempferol and quercetin from different tea extracts taken after 30 min.

Sample concentrations:			
Tea	**Extraction solvent**	**Compound**	**[ug/ml] sample**
Earl Grey	Me	**Chrysin**	0.000
		Kaempferol	2.141

		Quercetin	6.388
Five Roses	H_2O	**Chrysin**	4.277
		Kaempferol	4.853
		Quercetin	24.291
	Me	**Chrysin**	11.884
		Kaempferol	0.000
		Quercetin	0.000
Rooibos	H_2O	**Chrysin**	1.347
		Kaempferol	0.416
		Quercetin	14.129
	Me	**Chrysin**	5.789
		Kaempferol	60.217
		Quercetin	167.423
Kilimanjaro	H_2O	**Chrysin**	1.099
		Kaempferol	1.160
		Quercetin	4.531
	Me	**Chrysin**	5.900
		Kaempferol	2.229
		Quercetin	0.000
Dilmah Ceylon	H_2O	**Chrysin**	0.535
		Kaempferol	0.739
		Quercetin	0.815
	Me	**Chrysin**	16.139
		Kaempferol	0.000
		Quercetin	0.000

The teas with highest to lowest C and Q concentrations using boiled water were Five roses®>Rooibos®>Kilimanjaro®>Dilmah Ceylon® tea. For K the order changed to Five Roses®>Kilimanjaro®>Dilmah Ceylon®>Rooibos tea®. Thus it can be concluded that more flavonoids are ingested by drinking Five Roses® tea than the others.

With the Me extractions, the only tea that did not have C present, was Earl Grey® tea. The order of extraction for C from highest to lowest was Dilmah Ceylon®>Five Roses®>Kilimanjaro®>Rooibos®>Earl Grey®. All of these values were higher than the ones achieved by using boiling water. Two of the teas did not have K present, namely Dilmah Ceylon®- and Five Roses® tea. This showed that the solvent used had a definite influence on extraction as Five Roses® had the best results for K extraction using boiling water. The order for K extraction was Rooibos®>Kilimanjaro®>Earl Grey® tea. This is of importance as

Rooibos® tea is local to South Africa, being a frequent beverage that is ingested by the local populace. Only two of the teas had Q present, being Rooibos®>Earl Grey®.

This showed for extraction purposes for research, better results could be obtained from extraction of Rooibos® by using Me. For better results to determine ingestion, it was better to use water as solvent. If these compounds were to be extracted from Me (which is toxic to humans), a suitable drying method needs to be implemented that does not show a loss in product or destruction of the compound.

No increase/decrease analysis is done as these were single extractions per sample and not done over a time period as in the case of the onion and broccoli extractions.

5.5 General discussion and conclusion

As this was the first time that this kind of study had been done in a South African setting, there were not any other results from this region to compare with. This led to comparisons being drawn between international studies done on these specific plants testing for C, K and Q. This made it more difficult, as mentioned in chapter 2 stating that different aspects could influence the concentrations of these different compounds found in the plants, being climate, cultivar, farming practices, geography, processing and storage conditions (Amiot *et al.*, 1995; Häkkinen *et al.*, 2000; Patil *et al.*, 1995). This is made more difficult as these specified conditions found in the South African setting could differ dramatically from international settings.

Upon initiation of the experiments, it was found that the “like dissolves like” principle was crucial to the selection of the solvent as it dramatically influenced the extraction process concentrations (Gupta *et al.*, 2012). This was demonstrated in the comparison between extract concentration quantities achieved with the tea extractions where water had a higher polarity and dielectric constant than Me (table 6.1.), indicating that higher solubility was achieved in water. This was indicative of the flavonoids’ polarity out of fresh extracts, especially if the glycosidic molecule was still attached to the aglycone (increasing the flavonoids polarity)(Torres *et al.*, 2005). Furthermore, as this extraction using water was not done at room temperature, the influence of temperature could not be accounted for. Thus it is suggested that further research be conducted to identify the importance of different temperatures on extraction of different flavonoids, using different solvents. Ultimately, the goal of the research determines the conclusion perspective, which in turn determines different factors of the study to be taken into account when selecting a solvent for use. In this case, it was ingestion by humans versus extraction for research purposes. For human

ingestion, boiled water achieved better results, compared to that of the Me extraction which in turn supplied better results for research purposes extractions.

It can be seen from the results of this study that the different preparation techniques influenced the concentrations of the various extracts. This could be attributed to the change in molecular structure as the plant material was bruised, crushed, liquefied etc. In the case where heat was used, extraction was promoted, until breakdown started occurring, as was evident from these results, especially with USE. This problem was also stated by Palma *et al.* (2001) who concluded that oxidation of flavonoids is accelerated when temperature is increased, without affecting recovery results (Søltoft *et al.*, 2009). Overall, the best extraction results were achieved by MAE, followed by maceration, USE and then the WBE; as seen when comparing the concentrations of the different methods. This agrees with the Søltoft *et al.* (2009) study. Conventional extraction methods show an increased risk of degradation taking place because of the long extraction times, with the factors influencing it being light, air and temperature which cause the release of oxidative enzymes furthering degradation (Liazid *et al.*, 2007), especially as seen for C. For MAE, fresh samples were used, as well as with the grilled onions and cooked plant materials. It was seen that fresh plant material showed better extraction results than both the frozen plant material and the cooked plant material. Søltoft *et al.* (2009) stated that cellular structures could have been destroyed or damged during a freeze-drying process applied in their study, which also caused the concentrations of the analytes to be lower. This idea could also be applied to normal freezing procedures, especially as seen for K and C in this study. In the case of the grilled onions, some of the flavonoid content could have been obtained from the added butter or margarine content of the extraction, but this remains inconclusive. These results were discussed in more detail in the results section.

It was also observed that (in some cases) the greater the degree to which the plant material had been processed, the lower the concentration of the analytes in the extracts. This showed the result of concentrations being higher in the order of whole>cut>blended. In addition, in some cases, this order was completely reversed, depending on the plant, being the yellow onion, red onion or broccoli, as well as extraction procedure used (like MAE). For the specific plants, it seemed that better results were achieved in the order of red onions>yellow onions>broccoli. From the results obtained, it was seen that yellow onion plant material was more susceptible to temperature changes as it showed lower concentration of the flavonols in frozen samples than those of red onions and broccoli. For cooked samples, broccoli achieved better results for Q, followed by red onions and then yellow onions. This showed the difference and effect that an increase in temperature has, taking into account the results obtained from cooked and frozen samples, as the one started from a very low temperature, and the other a very high temperature.

As mentioned with MAE, it was seen that C is more susceptible to higher temperatures, showing degradation. Liazid, *et al.* (2007) also found that flavonols, especially K, showed a tendency to be stable under heated conditions of up to 125 °C, but that this stability decreased as the amount of OH-groups increased as seen in compared flavonols. This study's results differ from Liazid, *et al.* (2007) as their concluding statement acknowledged that the fewer the substituents on the aromatic rings, the more stable the compound is to degradation. In this case, C, having the least substituents, showed a greater degree of degradation than that of the flavonols which have more substituents.

Taking into consideration that the proposed daily intake for flavonoids should be around 20 mg/day for flavonols, with Q showing a daily intake of 10 mg/day (Bravo, 1998; Yao *et al.*, 2004), it could clearly be noted that this quantity would not be ingested when considering the results obtained for Q and K (both being flavonols) alone, or together. One study done comparing the flavonol intake in an international setting by comparing the daily intake of different countries, found the flavonol and flavone daily intake to range between 3.6 mg/day (Mexico) to 77 mg/day (Scandinavia). The mean intake recorded in this study was 27.6 mg/day with a standard deviation (SD) of 19.5 mg/day, taking into account fourteen different countries (de Vries *et al.*, 1997). For beneficial effects to manifest, the suggested intake is even higher than the daily intake, for example, as stated with C, that the daily intake should be between 0.5 g - 3 g to see a rise in testosterone level (Dean, 2004; Mani & Natesan, 2018). Therefore, this concludes that the concentrations obtained from these extractions are too low to achieve this effect. This amount would increase if the amount increases above the experimental weight used, but a single source of edible plant material will still not provide the advised flavonoid quantity for daily intake. It is thus rather suggested that the whole range of edible sources be incorporated in the person's daily diet. Although, that would make it more difficult to determine per individual.

From this studies results, it is suggested to take into consideration the total flavonoid content of the components being tested for. This is because the specific content of a specific component (C, K or Q) after a specific time could lead to misleading information regarding the total quantity flavonoids extracted at a certain point in the extraction process. The different extracts' concentrations added together for the three subsequent extracts using a specific method gives a better estimate of the flavonoid content present. For future use, this should be taken into consideration when determining the best extraction times to establish if optimal compound recovery from the different plant materials used has been achieved.

In conclusion, C, K and Q were found in all plant material, depending on the extraction method used. Onions proved to be a good source of Q and K as can be seen in other studies as well (Bilyk *et al.*, 1984; Bonaccorsi *et al.*, 2005; Lee *et al.*, 2008; Nuutila *et al.*, 2002; Søltoft *et al.*,

2009; Vian *et al.*, 2009). Previous studies have also found the presence of Q and K in broccoli (Koh *et al.*, 2009; Price *et al.*, 1999). Even out of these studies it could be seen that the quantities of flavonoids from these individual food sources were insufficient in reaching the daily intake estimated as mentioned above, as they had reworked their results to mg/kg plant material, concluding this study.

CHAPTER 6 CONCLUSION

6.1 Introduction

Interest in the use of flavonoids for the treatment of Alzheimer's disease and other illnesses has arisen during the past few years. As flavonoids are plant secondary metabolites, there are thousands of different compounds that have been identified as flavonoids (Bravo, 1998; Karuppagounder *et al.*, 2016; Mladěnka *et al.*, 2011; Ross & Kasum, 2002; Yang *et al.*, 2014). Among the most notable flavonoids are quercetin (Q) and kaempferol (K), which were investigated in this study. Both of these are part of the flavonol class of flavonoids. Furthermore, a less frequently studied flavonoid is chrysin (C), which is part of the flavanol group, was also included in this study. The flavonoids are known for their antioxidative potential and can scavenge the free radicals responsible for oxidative damage within the body (Doronicheva *et al.*, 2007; Harris *et al.*, 2006; Kandhare *et al.*, 2014; Smith & Luo, 2003). This could over time reduce oxidative damage in the brain that causes inflammation and further progression of tau proteins and amyloid β plaque formation (Croft, 1998; Cummings, 2001; Hardy & Selkoe, 2002; Jalbert *et al.*, 2008; Mawuenyega *et al.*, 2010; McKhann *et al.*, 1984; Roth *et al.*, 1999). This is discussed in more detail in chapter 2. This antioxidative effect of flavonoids can be seen in other parts of the body, as it was seen that these flavonoids may play a role in the treatment of cancer, vascular disease and a number of other diseases (Bravo, 1998; Erlund *et al.*, 2000; Knekt *et al.*, 1996; Ross & Kasum, 2002).

6.2 Findings and conclusion

In the introduction (Chapter 1) it was postulated that an extraction and analysis method for the separation of the flavonoids, chrysin, kaempferol and quercetin, could be developed as based on previous methods developed by Bimakr *et al*. (2011), Kim *et al*. (2002), Martino & Guyer (2004), Oniszczuk *et al*. (2016), Roldán-Marín *et al*. (2009) and Vian *et al*. (2009). The hypothesis was proved as the method that was developed was suitable for these three flavonoids, and not only for a single flavonoid as has been done in the previous studies. This method allowed us to analyse the flavonoid content of different vegetables that are common in the South African diet, such as broccoli and onions.

In chapter 3, a simple method for preliminary screening using TLC plates was developed, thus achieving the screening objective for this study. The TLC conditions consisted of two final mobile phases (MPs) that were derived from previous studies and adjusted to suit this study. The MPs are a) toluene 80%: ethanol 20% and b) toluene 60%: ethyl acetate 30%: formic acid 10%. The

selection of MP is left to the analyst's discretion and availability of chemicals, as well as the goal of the study.

In chapter 4, an HPLC method that was derived from previous studies was adjusted for specific analysis of our compounds, C, K and Q. The MP used consisted of 10 mM phosphoric acid (H_3PO_4) 50%: methanol 25%: acetonitrile 25%. The flow rate was set to 1 ml/min, the wavelength of detection to 280 nm. The analytical column was a Kinetex® EVO C_{18} column (250 mm x 4.6 mm; 5 µm particle size and 100 Å pore size). This method was qualitatively validated and proved useful in identification of the different compounds tested for, C, K and Q. This addressed the objective for the establishment of an HPLC analysis method, and validation of this method. This proved a useful tool as the extracts obtained from the extraction methods used on the processed onions and broccoli could be directly compared using the retention times of the different standards for identification (discussed in chapter 5). This method was used to give an estimate of the concentrations of C, K and Q in the extracts, thus reaching the objective of quantifying the compounds in plant material. These analyses were carried out for different samples obtained by various extraction methods. The extraction methods were waterbath extraction, ultrasonic extraction, maceration and microwave assisted extraction. Thus the final objective was reached which aimed at finding a suitable extraction method. The most efficient method proved to be microwave extraction which also had the shortest extraction time of 60 s in total. Waterbath and ultrasonication both had extraction times that lasted for 2 hours and maceration required the longest time, 8 hours. All methods proved to be effective in extraction of the flavonoid compounds, but showed great variability.

Overall it may be concluded that the flavonoid content of the vegetables considered in this study is not sufficient to ensure the required ingestion of these compounds. As seen from this study's estimated quantities and discussed in the conclusion of chapter 5, the quantities do not measure up to the daily intake content of the values obtained by de Vries, *et al.* (1997), which ranged from 3.6 mg/day to 77 mg/day. For this reason, if these results are lower than the daily amount being ingested generally, this will not be sufficient in obtaining quantities beneficial to achieve specific health goals as the general daily intake is lower than the suggested quantities as seen in the example provided in chapter 5 (Dean, 2004), being 0.5 g to 3 g. C, K and Q, and other flavonoids would need to be obtained from more than one source, showing the importance of a balanced diet. While these vegetables provide flavonoids that may have beneficial effects for Alzheimer's disease, it should be noted that these vegetables should rather be used in conjunction with pharmacological therapy, and not as sole treatment. This emphasises the importance of a healthy lifestyle consisting of an adequate diet, exercise, mental stimulation and pharmacological therapy.

6.3 Recommendations for future studies

1. A fully validated method development could be done in future studies applicable to digestible plant material.
2. More reliable results could be achieved if a future study is repeated in duplicate or triplicate, rather than single extractions of the plant material. In such an instance, it would be advisable to focus on the extraction method that proved to be most efficient in this study.
3. Based on the degradation of the three analytes observed in this study, the effect of temperature over time should be investigated to establish degradation tempo and stability for future studies.
4. Assessment of the effect of temperature on flavonoids could prove interesting.
5. Future studies may analyse other flavonoids not considered in this study.
6. Assessing if isomerisation occurs for the conversion of K to Q could be an important future consideration.

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Addendum A

The following are the concentration tables that shows the estimated concentrations of the different extractions.

Fresh results:

Table A.1, A.2, A.3 and A.4 shows the calculated concentrations for fresh plant material using area under the curve (AUC) compared to the AUC of the standards. The AUC of the mixed standard sample (M) was compared to a 1 mg/ml standard.

Table A.1 shows the results for fresh yellow onion plant material:

Table A.1: Calculated concentrations of extraction samples as time progressed for fresh yellow onion plant material:

Sample concentrations:					
Extraction method	**Sample**	**Compound**	**[µg/ml] sample 1**	**[µg/ml] sample 2**	**[µg/ml] sample 3**
Yellow onion					
Maceration	**Whole**	**Chrysin**	67.421	16.714	4.967
		Kaempferol	5.756	4.019	5.298
		Quercetin	4.915	9.238	36.539
	Cut	**Chrysin**	1.484	0.541	0.482
		Kaempferol	2.433	1.159	1.286
		Quercetin	4.400	20.187	34.174
	Blended	**Chrysin**	0.427	0.384	0.358
		Kaempferol	0.862	0.827	1.673
		Quercetin	9.838	2.961	2.531
Water bath	**Whole**	**Chrysin**	1.286	0.484	0.574
		Kaempferol	0.918	1.048	1.245
		Quercetin	13.949	1.532	5.534
	Cut	**Chrysin**	0.326	0.366	0.210
		Kaempferol	0.804	1.146	1.315
		Quercetin	1.332	0.522	8.600
	Blended	**Chrysin**	22.448	14.506	2.695
		Kaempferol	3.635	3.008	1.501
		Quercetin	147.161	21.341	20.914
Ultrasonication	**Whole**	**Chrysin**	0.411	0.488	0.000

		Kaempferol	1.304	1.587	1.695
		Quercetin	39.170	1.451	48.067
	Cut	**Chrysin**	0.256	0.309	0.302
		Kaempferol	1.366	2.488	0.922
		Quercetin	10.339	17.391	3.463
	Blended	**Chrysin**	0.508	0.271	0.355
		Kaempferol	1.170	1.067	1.388
		Quercetin	16.822	30.658	25.207

Table A.2: Calculated concentrations of extraction samples as time progressed for fresh red onion plant material.

Sample concentrations:					
Extraction method	**Sample**	**Compound**	**[µg/ml] sample 1**	**[µg/ml] sample 2**	**[µg/ml] sample 3**
Red onion					
Maceration	**Whole**	**Chrysin**	11.504	9.692	20.895
		Kaempferol	9.907	2.688	3.649
		Quercetin	79.930	3.076	11.385
	Cut	**Chrysin**	5.204	1.050	5.971
		Kaempferol	2.037	1.130	2.133
		Quercetin	2.596	1.718	14.150
	Blended	**Chrysin**	1.314	0.398	1.356
		Kaempferol	1.210	0.896	1.734
		Quercetin	3.131	1.135	2.339
Water bath	**Whole**	**Chrysin**	0.452	0.355	0.272
		Kaempferol	0.506	0.803	0.882
		Quercetin	1.364	8.787	6.883
	Cut	**Chrysin**	0.243	0.000	0.242
		Kaempferol	1.181	0.000	1.131
		Quercetin	0.635	2.722	6.710
	Blended	**Chrysin**	23.642	7.000	1.683
		Kaempferol	8.126	1.348	0.986
		Quercetin	9.631	2.230	13.381
Ultrasonication	**Whole**	**Chrysin**	0.544	1.062	0.337
		Kaempferol	0.842	1.459	0.939
		Quercetin	11.662	13.566	6.419
	Cut	**Chrysin**	0.373	0.114	0.259
		Kaempferol	0.814	2.066	1.111
		Quercetin	7.428	1.712	3.348

	Blended	**Chrysin**	0.236	0.228	0.538
		Kaempferol	1.032	1.133	1.080
		Quercetin	9.381	7.313	4.614

Table A.3: Calculated concentrations of extraction samples as time progressed for fresh broccoli plant material.

Sample concentrations:					
Extraction method	**Sample**	**Compound**	**[µg/ml] sample 1**	**[µg/ml] sample 2**	**[µg/ml] sample 3**
Broccoli					
Maceration	**Whole**	**Chrysin**	6.004	0.000	1.352
		Kaempferol	1.820	0.321	1.775
		Quercetin	2.528	12.907	36.481
	Blended	**Chrysin**	0.000	1.598	1.221
		Kaempferol	0.497	0.967	0.583
		Quercetin	0.152	6.285	22.376
Water bath	**Whole**	**Chrysin**	0.000	0.162	0.000
		Kaempferol	0.527	0.700	0.000
		Quercetin	1.452	14.492	0.000
	Blended	**Chrysin**	0.340	0.000	0.000
		Kaempferol	0.360	0.408	0.400
		Quercetin	18.954	0.000	0.000
Ultrasonication	**Whole**	**Chrysin**	18.965	1.784	3.197
		Kaempferol	3.609	1.835	1.858
		Quercetin	30.846	17.151	15.469
	Blended	**Chrysin**	0.253	1.057	0.000
		Kaempferol	0.096	0.937	0.329
		Quercetin	11.658	0.733	1.448

Table A.4: Calculated concentrations of extraction samples as time progressed for fresh florets plant material.

Sample concentrations:					
Extraction method	**Sample**	**Compound**	**[µg/ml] sample 1**	**[µg/ml] sample 2**	**[µg/ml] sample 3**
Florets					
Maceration	**Whole**	**Chrysin**	0.362	0.353	0.168

		Kaempferol	0.660	0.363	0.166
		Quercetin	8.920	4.313	0.651
	Blended	**Chrysin**	0.040	0.430	0.352
		Kaempferol	0.000	0.764	0.552
		Quercetin	1.459	3.333	5.528
Water bath	**Whole**	**Chrysin**	0.496	0.331	0.517
		Kaempferol	0.586	0.576	0.709
		Quercetin	26.369	20.382	4.924
	Blended	**Chrysin**	5.252	1.197	1.506
		Kaempferol	4.882	2.176	1.568
		Quercetin	21.791	0.000	0.000
Ultrasonication	**Whole**	**Chrysin**	1.215	2.260	0.000
		Kaempferol	0.855	4.562	0.558
		Quercetin	0.000	0.000	13.900
	Blended	**Chrysin**	2.170	0.000	0.000
		Kaempferol	2.043	2.428	5.289
		Quercetin	36.833	75.041	47.653

Frozen results:

Table A.5, A.6, A.7 and A.8 shows the calculated concentrations of frozen plant material using AUC compared to the AUC of the standards. The AUC of M was compared to a 1 mg/ml standard.

Table A.5: Calculated concentrations of extraction samples as time progressed for frozen yellow onion plant material.

Sample concentrations:					
Extraction method	**Sample**	**Compound**	**[µg/ml] sample 1**	**[µg/ml] sample 2**	**[µg/ml] sample 3**
Yellow Onion					
Maceration	**Whole**	**Chrysin**	6.260	1.478	1.017
		Kaempferol	5.344	3.182	1.381
		Quercetin	16.052	9.107	7.472
	Cut	**Chrysin**	0.333	0.334	0.330
		Kaempferol	0.000	0.000	0.000
		Quercetin	2.315	4.588	2.108
	Blended	**Chrysin**	0.290	0.262	0.121
		Kaempferol	0.000	0.000	0.000
		Quercetin	3.111	2.447	1.292
Water bath	**Whole**	**Chrysin**	1.107	0.329	0.521

		Kaempferol	2.328	0.000	0.630
		Quercetin	5.953	3.727	5.044
	Cut	**Chrysin**	0.073	0.116	0.372
		Kaempferol	0.000	0.000	0.000
		Quercetin	0.000	1.963	2.494
	Blended	**Chrysin**	0.401	0.315	0.227
		Kaempferol	0.000	0.000	0.000
		Quercetin	10.352	0.000	0.000
Ultrasonication	**Whole**	**Chrysin**	0.188	0.096	0.476
		Kaempferol	0.000	0.000	0.832
		Quercetin	7.965	3.176	1.430
	Cut	**Chrysin**	0.216	0.077	0.091
		Kaempferol	0.000	0.000	0.000
		Quercetin	0.000	0.565	1.010
	Blended	**Chrysin**	2.339	3.027	2.394
		Kaempferol	2.818	2.921	2.264
		Quercetin	10.827	7.432	6.395

Table A.6: Calculated concentrations of extraction samples as time progressed for frozen red onion plant material.

Sample concentrations:					
Extraction method	**Sample**	**Compound**	**[µg/ml] sample 1**	**[µg/ml] sample 2**	**[µg/ml] sample 3**
Red Onion					
Maceration	**Whole**	**Chrysin**	3.015	6.325	0.529
		Kaempferol	0.000	2.526	0.000
		Quercetin	9.449	21.881	20.184
	Cut	**Chrysin**	0.000	0.337	0.000
		Kaempferol	1.966	1.518	2.602
		Quercetin	1.3345	28.414	16.901
	Blended	**Chrysin**	0.000	0.000	0.000
		Kaempferol	0.811	1.466	0.924
		Quercetin	8.381	8.527	6.314
Water bath	**Whole**	**Chrysin**	0.199	0.112	0.626
		Kaempferol	0.000	0.000	1.513
		Quercetin	7.464	6.537	20.496
	Cut	**Chrysin**	0.127	0.145	0.603

		Kaempferol	0.940	2.263	0.610
		Quercetin	1.235	3.162	2.116
	Blended	**Chrysin**	0.449	0.231	0.445
		Kaempferol	2.456	0.853	1.341
		Quercetin	4.450	1.305	14.591
Ultrasonication	**Whole**	**Chrysin**	0.000	0.000	0.000
		Kaempferol	0.000	1.016	1.618
		Quercetin	17.795	33.501	85.102
	Cut	**Chrysin**	0.452	0.671	0.282
		Kaempferol	0.587	1.019	0.893
		Quercetin	3.643	5.720	11.888
	Blended	**Chrysin**	0.295	0.348	0.237
		Kaempferol	1.491	1.540	0.383
		Quercetin	1.753	2.782	0.722

Table A.7: Calculated concentrations of extraction samples as time progressed for frozen broccoli plant material.

Sample concentrations:					
Extraction method	**Sample**	**Compound**	**[µg/ml] sample 1**	**[µg/ml] sample 2**	**[µg/ml] sample 3**
Broccoli					
Maceration	**Whole**	**Chrysin**	0.393	0.421	0.544
		Kaempferol	1.522	2.863	1.846
		Quercetin	1.374	0.000	3.674
	Blended	**Chrysin**	0.499	0.496	0.184
		Kaempferol	1.226	1.712	0.000
		Quercetin	1.269	0.810	20.671
Water bath	**Whole**	**Chrysin**	25.528	4.976	3.322
		Kaempferol	5.799	1.874	2.723
		Quercetin	4.590	4.039	15.528
	Blended	**Chrysin**	0.548	0.678	0.279
		Kaempferol	2.859	0.746	1.385
		Quercetin	1.479	2.661	0.000
Ultrasonication	**Whole**	**Chrysin**	0.603	0.297	0.456
		Kaempferol	2.140	0.580	0.793
		Quercetin	2.288	10.325	4.117
	Blended	**Chrysin**	0.093	0.210	0.626

		Kaempferol	0.000	2.025	1.189
		Quercetin	3.838	1.847	2.931

Table A.8: Calculated concentrations of extraction samples as time progressed for frozen florets plant material.

Sample concentrations:					
Extraction method	**Sample**	**Compound**	**[µg/ml] sample 1**	**[µg/ml] sample 2**	**[µg/ml] sample 3**
Florets					
Maceration	**Whole**	**Chrysin**	2.279	11.542	7.767
		Kaempferol	15.822	10.691	5.313
		Quercetin	49.052	61.352	37.961
	Blended	**Chrysin**	2.201	0.407	0.581
		Kaempferol	33.463	1.103	17.487
		Quercetin	23.502	12.513	25.354
Water bath	**Whole**	**Chrysin**	0.187	0.216	0.253
		Kaempferol	1.748	1.728	2.458
		Quercetin	8.261	0.000	12.805
	Blended	**Chrysin**	0.815	0.897	0.419
		Kaempferol	0.924	0.879	0.441
		Quercetin	1.749	6.945	0.000
Ultrasonication	**Whole**	**Chrysin**	5.294	2.723	0.915
		Kaempferol	2.549	4.352	0.903
		Quercetin	11.615	25.044	17.702
	Blended	**Chrysin**	0.800	0.405	0.462
		Kaempferol	8.331	4.374	0.814
		Quercetin	11.292	5.930	1.371

MAE results:

Table A.9, A.10, A.11 and A.12 shows the calculated concentrations using AUC compared to the AUC of the standards. The AUC of the mixed standard samples (M) was compared to a 1 mg/ml standard with which a percentage was calculated. The concentrations of the Q, K and C could

thus be calculated by comparing the AUC of the samples to the AUC of the M. For the MAE 60% power and 80% power was used, which is related back to 900 W and 1 200 W individually.

Table A.9: Calculated concentrations of extraction samples as time progressed for microwaved yellow onion plant material.

Sample concentrations:					
Preparation	**Method**	**Compound**	**[µg/ml] sample 1**	**[µg/ml] sample 2**	**[µg/ml] sample 3**
Yellow onion					
Whole	**Mi 60**	**Chrysin**	15.473	64.546	22.559
		Kaempferol	19.931	77.835	45.919
		Quercetin	14.832	59.046	36.386
Whole	**Mi 80**	**Chrysin**	10.166	24.530	7.096
		Kaempferol	12.877	55.899	31.749
		Quercetin	9.995	43.749	24.889
Cut	**Mi 60**	**Chrysin**	6.474	13.119	0.853
		Kaempferol	10.809	56.684	3.124
		Quercetin	8.654	113.497	49.919
Cut	**Mi 80**	**Chrysin**	4.637	24.474	0.848
		Kaempferol	15.852	25.910	2.085
		Quercetin	0.601	23.223	5.228
Blended	**Mi 60**	**Chrysin**	0.000	0.000	7.174
		Kaempferol	0.228	0.236	13.563
		Quercetin	1.826	3.096	17.968
Blended	**Mi 80**	**Chrysin**	1.043	4.411	0.000
		Kaempferol	1.179	9.060	0.731
		Quercetin	7.060	22.402	10.781

Microwave power at 60% (Mi 60); Microwave power at 80% (Mi 80).

Table A.10: Calculated concentrations of extraction samples as time progressed for microwaved red onion plant material.

Sample concentrations:					
Preparation	**Method**	**Compound**	**[µg/ml] sample 1**	**[µg/ml] sample 2**	**[µg/ml] sample 3**
Red onion					
Whole	**Mi 60**	**Chrysin**	2.311	3.153	45.302
		Kaempferol	0.000	1.000	14.381
		Quercetin	22.935	50.712	116.121

Whole	**Mi 80**	**Chrysin**	2.227	0.000	2.356
		Kaempferol	5.594	0.382	5.112
		Quercetin	10.317	3.278	4.731
Cut	**Mi 60**	**Chrysin**	0.000	1.653	0.357
		Kaempferol	0.962	3.952	4.481
		Quercetin	14.583	84.184	125.878
Cut	**Mi 80**	**Chrysin**	5.264	1.341	0.000
		Kaempferol	8.820	3.915	1.705
		Quercetin	12.766	147.655	96.390
Blended	**Mi 60**	**Chrysin**	0.000	1.131	1.830
		Kaempferol	0.000	0.000	0.000
		Quercetin	12.235	11.687	18.572
Blended	**Mi 80**	**Chrysin**	7.013	8.333	2.966
		Kaempferol	11.050	7.233	3.951
		Quercetin	23.098	23.866	26.755

Microwave power at 60% (Mi 60); Microwave power at 80% (Mi 80).

Table A.11: Calculated concentrations of extraction samples as time progressed for microwaved broccoli plant material.

Sample concentrations:					
Preparation	**Method**	**Compound**	**[µg/ml] sample 1**	**[µg/ml] sample 2**	**[µg/ml] sample 3**
Broccoli					
Whole	**Mi 60**	**Chrysin**	7.321	0.238	0.980
		Kaempferol	2.558	2.614	3.115
		Quercetin	6.050	2.085	8.234
Whole	**Mi 80**	**Chrysin**	0.000	0.310	0.584
		Kaempferol	0.000	3.586	5.235
		Quercetin	0.000	17.234	60.149
Blended	**Mi 60**	**Chrysin**	0.550	0.628	1.112
		Kaempferol	2.483	3.059	5.270
		Quercetin	0.000	2.869	20.151
Blended	**Mi 80**	**Chrysin**	0.703	0.813	0.737
		Kaempferol	2.949	3.567	4.238
		Quercetin	26.566	22.454	10.536

Microwave power at 60% (Mi 60); Microwave power at 80% (Mi 80).

Table A.12: Calculated concentrations of extraction samples as time progressed for microwaved broccoli plant material.

Sample concentrations:					
Preparation	**Method**	**Compound**	**[µg/ml] sample 1**	**[µg/ml] sample 2**	**[µg/ml] sample 3**
Florets					
Whole	**Mi 60**	**Chrysin**	1.463	45.524	3.957
		Kaempferol	2.158	9.597	5.647
		Quercetin	3.531	6.806	19.621
Whole	**Mi 80**	**Chrysin**	0.000	1.638	0.847
		Kaempferol	0.000	5.484	6.576
		Quercetin	0.000	2.653	3.293
Blended	**Mi 60**	**Chrysin**	1.744	0.396	0.769
		Kaempferol	23.195	13.880	12.337
		Quercetin	30.469	27.466	27.047
Blended	**Mi 80**	**Chrysin**	1.008	0.232	0.983
		Kaempferol	7.757	10.865	10.937
		Quercetin	12.151	14.509	15.517

Microwave power at 60% (Mi 60); Microwave power at 80% (Mi 80).