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Development and application of flavonoid intake assessment methods and the impact of flavonoids on cognitive and physical outcomes

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(Caldwell)

"This thesis is presented as part of the requirements for the award of the Degree of Doctor of Philosophy from the University of Wollongong"

2015

CERTIFICATION

I, Katherine Kent, declare that this thesis, submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Medicine, Faculty of Science, Medicine and Health, University of Wollongong, is wholly my own work unless otherwise references or acknowledged. This document has not been submitted for qualifications at any other academic institution.

Katherine Kent
19 October 2016

STATEMENT OF VERIFICATION

This statement verifies that the greater part of the work in the above named manuscripts is attributed to the candidate, Katherine Kent, under the guidance of her supervisors, took primary responsibility for the study design, all data collection and analysis and prepared the first draft of each manuscript. She then responded to editorial suggestions of co-authors and prepared the articles for submission to the relevant journals.

Katherine Kent (PhD Candidate)

Associate Professor Karen Charlton (Primary Supervisor)

DEDICATION

To Matt and Harry

ABSTRACT

A growing body of evidence suggests that a diet rich in flavonoids, naturally occurring plant-based compounds, is associated with improved health outcomes. However, current knowledge relating to the measurement of dietary flavonoid intake is limited and studies assessing the association of flavonoid-rich food supplementation with cognitive and physical health outcomes are scarce. This doctoral thesis outlines six studies that were developed to address two main research questions. Firstly, what methods should be employed to measure flavonoid intake? Secondly, how does the consumption of dietary flavonoid impact cognitive and physical outcomes?

In order to associate the impact of dietary flavonoids on health outcomes, accurate dietary assessment is fundamental. A systematic literature review evaluates the various methods employed by current literature to measure flavonoid intake. The review emphasises the reliance of studies utilising unsound dietary assessment methods to measure flavonoid intake and demonstrates that few studies utilise objectives biomarkers as a measure of flavonoid intake.

Dietary flavonoid intake estimates in Australia are limited, and specifically the estimations of flavonoid intake in older Australians are inadequate. A Food Frequency Questionnaire for the measurement of dietary flavonoid intake in this cohort was developed, based on secondary data analysis of detailed dietary information in older adults. The tool is validated against food records and assessed for reliability in a group of older Australians, showing to be a relatively useful instrument to measure flavonoid intake.

In response to the lack of biomarker data utilised in current flavonoid research, a sensitive and specific GC-MS method was developed to determine flavonoids and phenolic acids in biological samples. The developed method has potential to measure 115 flavonoid-related biomarkers in various biological samples, and is applied in later studies of this thesis.

A second systematic literature review identifies a paucity of research regarding food-based anthocyanin (a subclass of flavonoids) consumption and cognitive outcomes. Various methodological limitations in published studies are highlighted, including a lack of information regarding dosage and dose-timings, age related variations in responses and small sample study sizes. The findings are utilised inform the development of acute and longer-term intervention studies to assess the impact of a flavonoid and anthocyanin-rich cherry juice supplementation on cognitive and physical outcomes.

A pilot cross-over study assesses the acute effects on cognition, blood pressure and plasma biomarkers associated with consumption of a 300ml cherry juice, provided either as a single quantity or as 3x100ml doses administered over 2h. In young adults, older adults, and older adults with dementia similarly, results indicated that the acute impact of anthocyanin-rich cherry juice consumption on cognition was not supported, but a dose-timing administration effect may influence blood pressure outcomes.

A longer-term intervention assesses whether daily consumption of anthocyanin-rich cherry juice changed cognition and blood pressure in older adults with dementia over 12-weeks. The results indicate that anthocyanin-rich cherry juice consumption improve cognitive performance and significantly reduced blood pressure. This study provides the first evidence that a feasible serving of flavonoid-rich food may have beneficial consequences in older adults with Alzheimer's type dementia

Overall, this doctoral thesis provides a number of contributions to the literature. Primarily it demonstrates that the dietary flavonoid intake measurement strategies are lacking and the novel tools developed by this thesis improve upon current methods with potential application in future research. Additionally, the findings of the flavonoid intervention trials support the indication that flavonoid, and especially anthocyanin-rich food consumption, may be beneficial for cognitive and physical outcomes.

PUBLICATIONS CONSTITUTING THIS THESIS

The chapters of this thesis have been prepared for publication as follows (*denotes maiden name):

PEER REVIEWED PUBLICATIONS

Appendices A-E

1. Caldwell* K, Charlton KE, Roodenrys S, Jenner A. 2015. Anthocyanin-rich cherry juice does not improve acute cognitive performance on RAVLT. Nutritional Neuroscience. DOI: 10.1179/1476830515Y.0000000005
2. Kent, K, Charlton K, Roodenrys S, Batterham M, Potter J, Traynor V, Gilbert H, Morgan O, Richards R, 2015. Consumption of anthocyanin-rich cherry juice for 12 weeks improves memory and cognition in older adults with mild to moderate dementia. European Journal of Nutrition DOI: 10.1007/s00394-015-1083-y
3. Kent, K, Charlton KE, Russell J, Mitchell P, Flood V, 2015. Estimation of flavonoid intake in older Australians: secondary data analysis of the Blue Mountains Eye Study. Journal of Nutrition in Gerontology and Geriatrics. 34 (4), 388-398. DOI: 10.1080/21551197.2015.1088917
4. Kent, K, Roodenrys, S, Charlton, KE, Gilbert H, Morgan O, Richards R 2016. Dietary flavonoid intake and cognitive performance in older adults with Alzheimer's type dementia. Journal of Aging: Research and Clinical Practice. 5(2):93-97
5. Kent, K, Charlton, KE, Jenner, A, Roodenrys, S, 2015. Acute reduction in blood pressure following consumption of anthocyanin-rich cherry juice may be dose-interval dependant: a pilot cross-over study. International Journal of Food Sciences and Nutrition, 67 (1), 47-52.

FULL CONFERENCE PAPER

Appendix F

1. Caldwell*, K. Analysing dietary flavonoid intake: methods, limitations and implications for research in ageing. 13th National Conference of Emerging Researchers in Ageing, Adelaide, SA, Australia, 24th – 25th November 2014. In print.

MANUSCRIPTS UNDER REVIEW

1. Kent, K, Charlton, K, Netzel, M, Fanning, K 2015. The impact of food-based anthocyanin consumption on cognition in human intervention trials: a systematic review. Under review at the Journal of Human Nutrition and Dietetics.

MANUSCRIPTS IN PREPARATION

1. Kent, K, Probst, Y, Guan, V, 2015. Evaluation of tools used for estimating dietary phytochemical intake: A systematic review. To be submitted to Journal of Nutrition, Health and Aging.

2. Kent, K, Charlton, KE, 2015. Development and validation of a food-frequency questionnaire to measure flavonoid intake in older adults using food records and plasma biomarkers: the method of triads. To be submitted to European Journal of Clinical Nutrition.

CONFERENCE ABSTRACTS

1. Caldwell*, K. & Charlton, K. 2013. Dietary intake and major sources of flavonoids in older Australians with Alzheimer's type dementia. Enabling Active Ageing Conference Proceedings: The 12th National Conference of Emerging Researchers in Ageing (pp. 44-44). 25 and 26 of November 2013, Sydney, Australia. Australia: Emerging Researchers in Ageing Australia.

2. Caldwell* K., Charlton K., Flood V., and Russell, J., 2014. Development of a Quantitative Food Frequency Questionnaire to Measure Flavonoid Intake in Older Australians. 24 and 25 of November 2013, Adelaide, Australia. Making Research Matter Conference Proceedings: The 13th National Conference of Emerging Researchers in Ageing (pp. 44-44).

3. Caldwell*, K., Charlton, K. E., Roodenrys, S. & Jenner, A. 2013. High anthocyanin cherry juice acutely impacts blood pressure but not cognition in young people, older people and dementia patients. Abstract presented at the 35th ESPEN Congress, 31 August 2013 - 03 September 2013, Leipzig, Germany. Clinical Nutrition, 32 (Supplement 1), S122.

4. Caldwell*, K., Charlton, K. E. & Roodenrys, S. 2012. The acute impact of high anthocyanin cherry juice on cognition and blood pressure in young people, older people and dementia patients. Annual Scientific Meeting of the Nutrition Society of Australia, 27-30 November 2012, Wollongong, Australia. Australasian Medical Journal, 5 (12), 723.

5. Caldwell*, K., Charlton, K. E. & Roodenrys, S. J. 2013. Estimation of dietary flavonoid intake and cognitive performance in older adults with Alzheimer's type dementia. The Nutrition Society of Australia and Nutrition Society of New Zealand 2013 Joint Annual Scientific Meeting, 4-6 December 2013, Brisbane, Australia. Australasian Medical Journal, 6 (11), 612.

6. Caldwell*, K., Charlton, K., Roodenrys, S., Batterham, M., Potter, J., Richards, R., Gilbert, H. & Morgan, O. 2014. The impact of fruit flavonoids from cherries on memory and cognition in older adults with mild to moderate dementia. NSA 2014 Annual Scientific Meeting, 26-28 November, Hobart, Australia. Journal of Nutrition and Intermediary Metabolism, 1 18-18.

OTHER PUBLICATIONS AND ABSTRACTS

The publications listed are not directly related to the outcomes of this thesis. However, the work was completed during the candidature and relates to the research skills built on by the candidate:

1. Charlton KE, Batterham MJ, Bowden S, Ghosh A, Caldwell* K, Potter J, Meyer B, Barone L, Milosavljevic M. A high prevalence of malnutrition in acute geriatric patients predicts adverse clinical outcomes and mortality at 12 months. Annual Scientific Meeting of the Nutrition Society of Australia, 27-30 November 2012.

2. Charlton, K. E., Batterham, M. J., Bowden, S., Ghosh, A., Caldwell*, K., Barone, L., Mason, M., Potter, J., Meyer, B. & Milosavljevic, M. 2013, 'A high prevalence of malnutrition in acute geriatric patients predicts adverse clinical outcomes and mortality within 12 months', e - SPEN Journal, vol. 8, no. 3, pp. e120-e125.

3. Traynor, V., Fernandez, R. & Caldwell*, K. 2013. The effects of spending time outdoors in daylight on the psychosocial wellbeing of older people and family carers: a comprehensive systematic review protocol. JBI Database of Systematic Reviews & Implementation Reports, 11 (9), 36-55.

4. Caldwell*, K., Fernandez, R., Traynor, V. & Perrin, C. 2014. Effects of spending time outdoors in daylight on the psychosocial well-being of older people and their family carers: a systematic review. The JBI Database of Systematic Reviews and Implementation Reports, 12 (9), 277-320.

5. Traynor, V., Fernandez, R. & Caldwell, K. 2013. What are the effects of spending time outdoors in daylight on the physical health of older people and family carers: a systematic review protocol. The JBI Database of Systematic Reviews and Implementation Reports, 11 (10), 76-88.

6. Caldwell, K., Fernandez, R. & Traynor, V. 2014. The effect of spending time outdoors in daylight on the psychosocial well-being of older people and their family carers. Australian Journal of Dementia Care, 3 (5), 25-25. Abstract of presentation from the Dementia Collaborative Research Centres' (DCRC) 2014 National Dementia Research Forum, Sydney, 19 September 2014.

7. Cusick, A., Heydon, M., Caldwell*, K. & Cohen, L. 2014. Finding measures of clinical placements quality for pre-service health services training: challenges of definition and search strategy construction. Health Services Research: Evidence-Based Practice Meeting, London, UK, 1-3 July, 2014. BMC Health Services Research, 14 (Suppl. 2), P24-1-P24-2.

MEDIA COVERAGE OF THESIS RELATED RESEARCH

- Illawarra Health and Medical Research Institute (IHMRI) quarterly newsletter
    - Summer 2011: Scholarships for dementia students
    - Winter 2012: IHMRI project investigates the benefits of cherry juice on cognition
    - Summer 2012: Flavoursome flavonoids under investigation
    - Winter 2013: Cherry juice study uncovers new opportunities
    - Spring 2013: Cherries on top
    - Spring 2013: Local donor supports dementia research
    - Summer 2013: Generosity of local donor recognised
    - Autumn 2014: Plum job - examining the health benefits of a novel Australian fruit
    - Spring 2014: Three Minute Thesis competition winners
- Illawarra Mercury Print Newspaper: 23/12/2014, Powering the brain by cherry
- Illawarra Mercury Online: http://www.illawarramercury.com.au/story/2785722/serving-of-cherries-a-day-boosts-memory/
- WIN news broadcast, June 2013, Cherry Juice trial at IHMRI
- ABC Illawarra Radio, 5th June 2014, Cherries and dementia
- ABC Illawarra website, 6th June 2014. A natural, effective dementia treatment with a cherry on top
- The Conversation, December 2015. Diet and dementia: The cherry on top!

PRIZES AND AWARDS

- Winner at Faculty of Science, Medicine and Health Three Minute Thesis competition 2014
- Runner Up, University of Wollongong Three Minute Thesis competition, 2014
- Australian Association of Gerontology Emerging Researchers in Ageing Travel Grant, 2014
- Nutrition Society of Australia Annual Scientific Meeting, Hobart Australia, Best Student Oral Presentation 2014
- Illawarra Health and Medical Research Institute (IHMRI) summer scholarship program for dementia research recipient 2011/2012
- School of Health Science scholarship funded delegate position to Students4Students Leadership Conference, Wollongong, Australia, 2011
- IHMRI awarded travel grant to attend an international conference, 2012
- Health and Behavioural Sciences travel grant to attend a national conference, 2012

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CHAPTER 1: INTRODUCTION

This chapter examines the scientific literature relevant to this thesis, introducing concepts related to the measurement of flavonoid intake and a broad overview of the assessment of flavonoid consumption on health outcomes. The introduction provides a background to the thesis and exposes the gaps in current literature, and relates these to the empirical chapters of this thesis. The main research questions are introduced and the methodologies applied to address these are presented.

1.

1.1 Flavonoids

Plant based foods form an integral component of the human diet and their consumption is consistently linked to the maintenance of health and the prevention of a vast array of diseases (1, 2). In the past, the compounds believed to be responsible for the protective effects of a plant-based diet included carotenoids and other antioxidant vitamins (1). More recently, a growing body of literature illustrates that other phytochemicals, which are non-nutritive compounds, significantly contribute to the antioxidant activity of fruits and vegetables and are consequently attributed to the observed health benefits (3).

These polyphenolic compounds, or polyphenols, encompass a huge range of compounds present in varying quantities in all plants and can be divided into the subclasses flavonoids, lignans, stilbenes and phenolic acids (4) (Figure 1-1). Flavonoids are further divided into six major classes: anthocyanins, flavanols, flavanones, flavones, flavonols and isoflavones (4) (Figure 1-1).

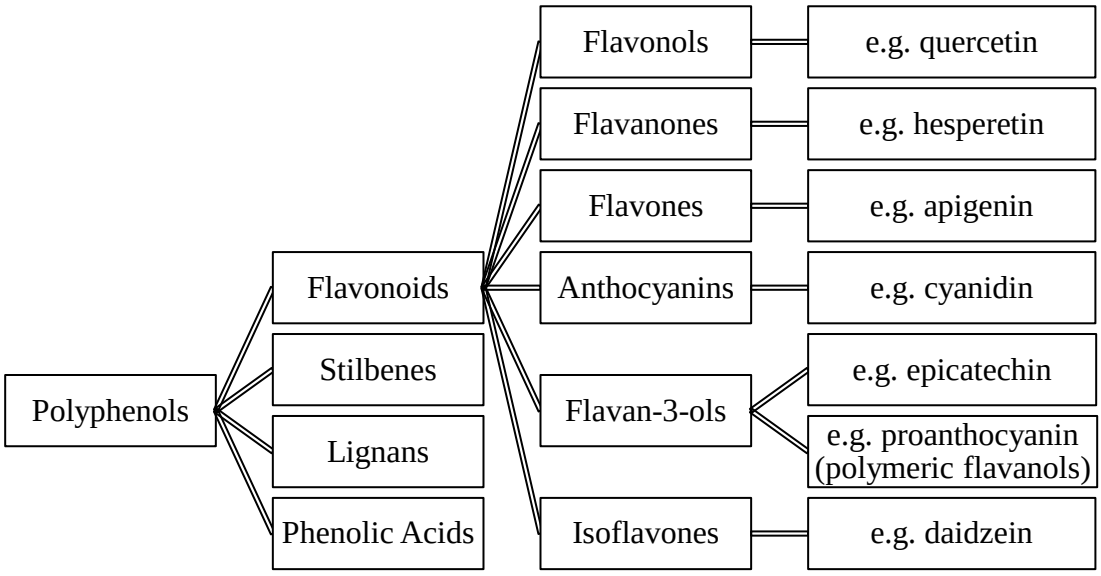

Figure 1-1 Classification of polyphenolic compounds and examples of flavonoids

Flavonoids have been studied intensely with several hundred flavonoids identified in foods. Flavonoids are found in particularly high concentrations in fruits and vegetables, wine, tea, cocoa, and soy (5). The consumption of flavonoids is estimated to be much higher than other dietary antioxidants; for example, around 10 fold higher than vitamin C, and 100 times higher than both vitamin E and carotenoids (6). Flavonoids are secondary plant metabolites, indicating they are not directly involved with the growth of plants, but their role is related to the protection of plants from microbe and insect damage (2). As a component of the human diet, flavonoids contribute to the sensory characteristics of foods such as flavour, astringency and colour (7).

1.2 Methods for measuring flavonoid intake

To link flavonoid consumption with positive health outcomes, the first and most fundamental step is to accurately estimate dietary flavonoid intake. Despite the first estimations of flavonoid intake being reported on a population level more than a decade ago, the numerous methods currently employed have evident flaws (8). As dietary flavonoid intake is difficult to quantify, numerous methods have been developed for application in various settings. Methods include various techniques within the fields of dietary assessment and biomarker analysis, as will be described below and in further detail in Chapter 2.

1.2.1 Methods of measuring flavonoid intake using dietary assessment

The most common method of estimating flavonoid intake in a population relies on dietary assessment and informatics (the development of computerised food composition databases (FCDB)). Generally, usual dietary analysis is performed using either a 24 hour diet recall, diet history or food record, which is then cross-referenced with a flavonoid specific FCDB. There are very few flavonoid specific FCDBs that exist, with the two most commonly used being the USDA Database for the Flavonoid Content of Selected Foods (9) and Phenol Explorer Database on Polyphenol Content in Foods (10). Aside from the limitations associated with each dietary assessment method, there are several well documented problems associated with utilising food composition databases to assign flavonoid content to foods to estimate dietary intake.

These limitations include inadequate food lists, and the inability of FCDBs to account for the specific growth and processing conditions that influence foods and the high inter-individual variation in the bioavailability of flavonoids. These limitations are suggested to have contributed to the large variations in estimations of flavonoid intake (11).

The limitations associated with current methods hinder the interpretation of observational research outcomes that associates dietary flavonoid intake and specific health outcomes. In light of Australia's expanding and ageing population and the need for lifestyle recommendations to improve healthy ageing, these limitations must be rectified to create advances in this research area. Most notably, there is currently little information available on the dietary intake of flavonoids in older Australians and no recommendations have been developed to advise the best way to measure flavonoid intake in this cohort using dietary assessment methods (this will be discussed in more detail in chapter 3 of this thesis).

1.2.2 Methods of measuring flavonoid intake using laboratory analysis

Dietary flavonoid intake can also be determined by quantifying biomarkers of flavonoid intake, which include flavonoids and phenolic acids found in biological samples (12). There are currently many methods of measuring flavonoid biomarkers in human biological samples. However, like dietary methodology, there is no standardised protocol of how to perform this analysis and researchers tend to develop and validate their own methods. Biomarkers of flavonoid intake are generally validated against dietary records (which capture either recent or habitual flavonoid intake) or after a dietary intervention study (13). A major limitation associated with accurately estimating flavonoid intake using biomarkers is that there are many hundreds of dietary flavonoids, which are rapidly and extensively metabolised into a wide range of metabolites (e.g. phenolic acids). There is currently no consensus regarding which of the intact flavonoid(s) or their in vivo phenolic acid metabolites are indicative of total dietary intake. Indeed, gaps in understanding of the exact uptake and metabolism of dietary flavonoids make it unclear as to which biological fluids

(plasma, urine or faecal water) to target for analysis of biomarkers. This concept will be addressed in more detail in chapter 4 of this thesis.

Another major limitation of these methods relates to the fact that they are physically invasive in terms of collecting biological specimens, which may especially be problematic to collect older adults and vulnerable groups. Moreover, most of the biomarker analyses are expensive and quite often it is not possible to perform them as part of a large epidemiological study (14). Future research should focus on identifying specific biomarkers of flavonoid intake and confirm the best methods in which to quantify these biomarkers in biological specimens in order to inform population research.

1.3 Dietary flavonoids and health outcomes

A high consumption of total flavonoids and flavonoid subclasses has been consistently linked with a protection against chronic diseases associated with ageing (15, 16), including cardiovascular (17) and neurodegenerative diseases (18). Biologically, flavonoids and their metabolites exert powerful antioxidant activities, influence vascular changes (17) and may guard areas of the brain against age-related decline (19, 20). The majority of research into the biological aspect of flavonoids has been pre-clinical. However, some strong epidemiological research exists, linking habitual flavonoid intake to various improved health outcomes in older adults, specifically cognition (21), coronary (22) and ischemic heart disease (23). Experimental trials in humans are rarer, but preliminary studies highlight positive results.

1.3.1 Dietary flavonoids and cardiovascular outcomes

Cardiovascular diseases (CVD), including stroke and coronary heart disease, are a major cause of mortality in developed countries and are closely associated with modifiable lifestyle factors including diet (24). A diet high in flavonoids has been linked with a reduction in CVD mortality (24, 25), with a recent review indicating four out of eight studies reporting a significant inverse association between coronary heart disease mortality with at least one flavonoid subclass (24). The association between flavonoid intake and incident stroke is less clear with three out of seven studies

reporting an association with flavonoid intake (24). The inconsistencies in epidemiologic studies associating flavonoid intake with CVD outcomes may relate to the use of different dietary assessment methods and flavonoid composition databases, differences in the types of flavonoid subclasses studied and differences between the populations (26).

The consumption of flavonoid-rich foods have been hypothesised to alter CVD risk factors including vascular function (27, 28) and blood pressure (29, 30), although the results are inconsistent. The mechanisms of action of flavonoids and cardiovascular outcomes are varied (25), with reviews indicating a multiplicity of effects (26). The antioxidant capacity of flavonoids has been postulated to reduce arterial stiffness through preventing the oxidation of LDL cholesterol (30). Another mechanism relates to a reduction in circulating free-radicals, reducing thrombogenesis and platelet aggregation in the body. The ability of flavonoids to modulate endothelial function, and thus influence blood pressure has also been investigated and relates to the modulation of nitric oxide bioavailability to the endothelium, resulting in endothelium-dependant vasodilation (27, 31, 32). Additional evidence suggests that flavonoid consumption may have the ability to prevent age-related vascular injury (33). It is possible that a culmination of all these effects may provide a protection against coronary heart disease and stroke.

Limitations in the literature surrounding flavonoid consumption and vascular outcomes relate to the limited research in human controlled trials, the inconsistencies with intervention (diet, pure flavonoids etc.), a lack of knowledge relating to timing and dose-dependency of outcomes and inconsistencies in outcomes between different population groups (34) (discussed in further detail in chapters 6 and 7 of this thesis).

1.3.2 Dietary flavonoids and cognition

The consumption of dietary flavonoids has been associated with improved cognitive performance and a preservation of cognitive function with ageing (35-37), with the flavonoid sub-groups flavanols, anthocyanins and flavanones having shown the most beneficial effects in the area of neuroprotection (20).

Initially, the biological actions of flavonoids on the brain were attributed solely to their antioxidant actions, ability to scavenge free radicals and reduction of inflammation in the body. It was then demonstrated that the antioxidant activity of flavonoids could not fully account for their total bioactivity in the brain as their concentration in brain tissue is very low, and their ability to cross the blood-brain barrier was questioned (38). Therefore, the actions of flavonoids in the brain have been investigated further and a variety of mechanisms have been postulated alongside their antioxidant activity. These include an ability to protect vulnerable neurons, increase the number of, and the strength of the connections between neurons (20), and an enhancement existing neuronal function (via the modulation of signalling processes)(39). Additionally, flavonoids have been shown to stimulate blood flow to the brain and induce neurogenesis in the hippocampus, the area of the brain associated with memory (38). The culmination of these mechanisms has been theorised as the potential pathways by which flavonoids may influence memory, learning and cognition and prevent neurodegeneration.

Limitations exist regarding the literature associating flavonoid intake with cognitive outcomes. Firstly, controlled intervention trials in humans are rare, with small samples sizes (40). Additionally, there is little consistency in the cognitive outcomes investigated across the literature, a wide range of intervention vehicles, doses and intervention durations investigated, which makes comparison and interpretation of study findings difficult (40) (discussed in further detail in chapters 6 and 7 of this thesis).

1.3.3 Dietary flavonoids and dementia

Neurodegenerative diseases is a term that describes a group of diseases that are irreversible and have debilitating symptoms including permanent memory impairment, continuous cognitive decline, and progressive behavioural disturbances (41). While cognitive impairment increases exponentially with age, it is not an inevitable consequence of ageing (42). Mild cognitive impairment and dementia related diseases are diagnosed when the level of impairment is greater than what is

associated with normal ageing (43). Dementia is the most common mental and behavioural disorder in Australia, accounting for 89% of cases in 2006 (44). In light of Australia's ageing population, it is predicted that by 2050 spending on dementia is set to exceed any other health condition if no significant developments in the prevention or treatment of this disease are made (45). It is therefore necessary to identify strategies that prevent or delay the onset of cognitive impairment and the role of nutrition in neuroprotection is a promising area of research.

Habitual consumption of flavonoid-rich foods and/or beverages has been associated with 50% reduction in the risk of developing a neurodegenerative disease (18) and a delay in the onset of Alzheimer's disease (46). The proposed mechanisms by which flavonoids might delay the initiation of dementia and slow the progression of dementia have been summarised in a recent review (19). The review suggests that flavonoids have potential to inhibit neuronal apoptosis (cell death) which is generated by neurotoxic species by reducing oxidative stress and neuroinflammation in the brain, the latter of which is a characteristic of dementia progression (19). Dietary flavonoids have also shown the potential to disrupt amyloid β aggregation and may inhibit the production of amyloid β through the disruption of amyloid precursor protein processing (19). However, much of this research relies on pre-clinical evidence, which cannot easily be translated into the human experience. To date, there has been little experimental research that investigates the impact of flavonoid rich foods on cognitive and physical functioning, specifically in older adults with a neurodegenerative disease. This may be a result of the difficulties in recruiting and retaining participants with dementia to clinical trials, which tend to have restrictive inclusion criteria, be intrusive and require large investments of time for participants (47).

The studies that have been conducted in healthy participants provide promising results in terms of cognitive benefits of flavonoids, for both acute and longer-term trials (48, 49). It is therefore surprising that studies in have not been extended to include older adults with a neurodegenerative disease. To the best of our knowledge, there are no human studies investigating the impact of dietary flavonoids on cognitive outcomes in older adults with a dementia, despite some small trials in older adults with mild-

cognitive impairment (50-52). A recent review (53) suggested that conclusions regarding the impact of dietary flavonoids on dementia cannot be made due to a lack of high quality research. It was recommended that future clinical trials be conducted, focussing on elucidating an effective dose to result in neuroprotective effects and the confirming bioavailability of flavonoids in population living with dementia (53) (addressed in further detail in chapters 6 and 7 of this thesis).

1.4 Summary of evidence and gaps in the literature

This overview of the literature has highlighted that there have been substantial recent advances in the measurement of dietary flavonoids and that preliminary studies support the impact of flavonoids on cognitive and physical outcomes. However, some major limitations of the current body of evidence are evident and there are a number of important questions still to be resolved surrounding dietary flavonoids.

Firstly, the measurement of dietary flavonoids, both with dietary assessment tools and with biomarkers of intake is not well established. Synthesis of the literature surrounding the current use of dietary tools to measure flavonoid intake and biomarker methods is needed to inform future research. Additionally, better methods to measure flavonoid intake through dietary assessment tools and biomarkers of intake need to be developed in light of the findings.

Secondly, the impact of flavonoid consumption on cognitive and physical outcomes needs be further investigated, with an emphasis on research in older adults and older adults with an existing neurodegenerative disease. While evidence exists for the potential beneficial effect of the consumption of flavonoids on a range of health outcomes, the body of evidence relating to flavonoid intake and improvements in cardiovascular and neurological health, especially for older adults with a neurodegenerative disease is sparse. There is a need to identify which cognitive domains are impacted by flavonoid intake and the most appropriate tools to measure the changes. The most notable gaps in the literature exist in relation to differences in short and long-term responses to flavonoid intake and dose-response relationships.

1.5 Aims and hypothesis

In light of the gaps in the literature surrounding flavonoids two main research questions were proposed (Figure 1-2):

1. What methods should be employed to measure flavonoid intake?
2. How does the consumption of dietary flavonoids impact cognitive and physical outcomes?

In order to effectively address these broad questions, six studies were developed (Figure 1-2).

Study 1: A systematic literature review that evaluates the tools used to estimate dietary flavonoid intake.
Study 2: Development and validation of a Food Frequency Questionnaire to determine dietary flavonoid intake.
Study 3: Development of a method to determine the phenolic profile of human plasma using Gas Chromatography/Mass Spectrometry technique.
Study 4: A systematic literature review that evaluates effect of anthocyanin-rich food intake on cognitive outcomes.
Study 5: An acute crossover study to assess the impact of differing doses of anthocyanin-rich cherry juice on cognitive and physical outcomes, and plasma phenolic profile.
Study 6: A randomised controlled trial to assess the longer-term impact of anthocyanin-rich cherry juice on cognition and physical outcomes in older adults, with mild to moderate dementia.

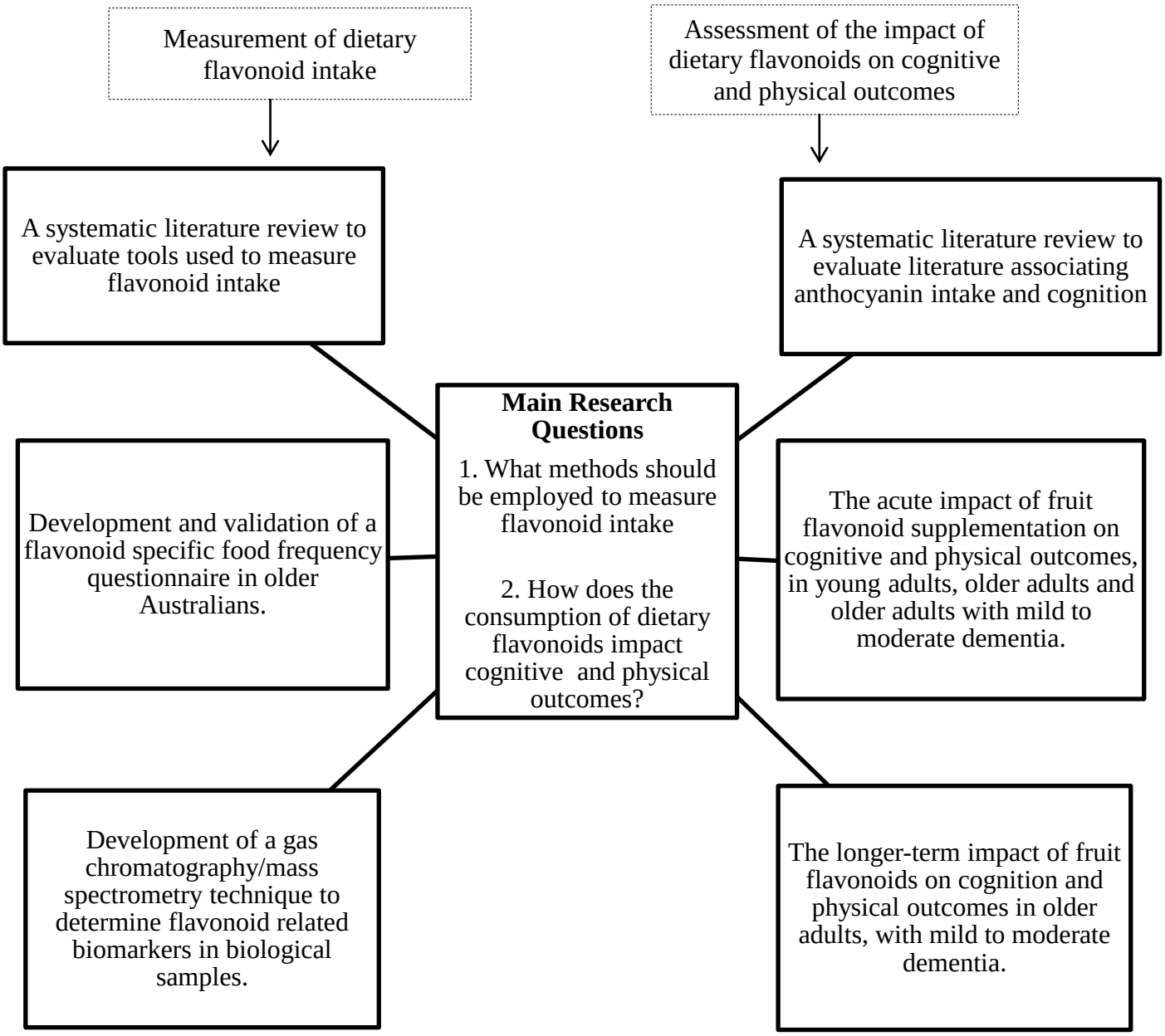

Figure 1-2 Overview of the thesis studies in relation to the central thesis questions

1.6 Thesis structure and methodology

In order to address the central research questions, several study designs need to be applied (Figure 1-2, Table 1-1). These approaches include various research methodologies, incorporating systematic literature reviews, dietary assessment methodologies, laboratory based methodologies and clinical trial study designs. The combination of research designs reflects the complexity of the central research questions. Each method is identified below, but significantly more detail pertaining to the methods of each study will be described in each chapter.

Chapter 1 provides an introduction to the literature surrounding this thesis, introduces each chapter and the methodology used throughout.

Chapter 2 is a systematic literature review that evaluates the tools used for estimating dietary flavonoid intake in current literature, to describe their application and relevance.

Chapter 3 and 4 describe the development of flavonoid intake assessment methods, with chapter 3 outlining a secondary data analysis of the Blue Mountains Eye Study to inform the development and validation of a food frequency questionnaire for older Australians, and chapter 4 describing the development of a novel gas chromatography/mass spectrometry technique to determine plasma phenolic profile in humans for application in chapter 6.

Chapter 5 is a systematic literature review that synthesises the literature relating to the impact of food-based anthocyanins on cognition, to inform the development of the studies comprising chapters 6 and 7.

Chapter 6 and chapter 7 introduce and provide the results of the human intervention trials conducted to assess the impact of fruit flavonoids on acute and longer-term cognition, blood pressure and physical outcomes.

Chapter 8 summarises the findings and generates recommendation for future research.

Each individual study comprising this thesis has been published or submitted for publication, but will be presented in this thesis in a consistent and cohesive format (with published papers included in the Appendices).

Table 1-1 Overview of thesis studies and study design

Thesis component	Study number (chapter number)	Study design utilised
Measurement of dietary flavonoid intake	1 (2)	Systematic literature review
	2 (3)	Secondary data analysis and dietary methodology validation study
	3 (4)	Laboratory methods development (GC/MS)
Assessment of the impact of flavonoids on cognition and physical outcomes	4 (5)	Systematic literature review
	5 (6)	Randomised acute crossover study
	6 (7)	Randomised controlled clinical trial

1.7 Significance of research

A review of the methodology used to measure flavonoid intake is important to evaluate the methods currently applied in flavonoid-related research and to provide recommendations for future research. With no gold standard method for measuring flavonoid intake, it is unclear which method for measuring or estimating dietary flavonoid intake is most useful in intervention and empirical studies. The systematic review provides an overview of the available strategies for measuring or estimating dietary flavonoid intake and may provide an important resource for researchers when developing future studies.

The development of new methodologies, both to estimate the intake of dietary flavonoids through dietary assessment and to confirm the uptake and metabolism of dietary flavonoids in biological samples, contributes significantly to a research area with few valid tool options.

The evaluation of current research that investigates the cognitive benefits associated with specific flavonoid rich foods is important to highlight the gaps in the literature and to provide recommendations for future research.

Investigations into the impact of anthocyanin-rich foods on acute and longer-term cognitive function and physical outcomes in specific population groups contributes original knowledge related to a body of literature surrounding the impact of flavonoids on cognition.

As a whole, this doctoral thesis provides a well-rounded approach to address the most significant gaps in current literature surrounding the measurement of flavonoid intake and the impact of flavonoids on cognitive and physical outcomes, with an emphasis on older adults with a neurodegenerative disease. It contributes to the fields of basic science, nutrition, psychology and public health, with each study contributing a novel and innovative investigation, which together, advances our scientific knowledge about dietary flavonoids.

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CHAPTER 2: EVALUATION OF TOOLS USED FOR ESTIMATING DIETARY FLAVONOID INTAKE: A SYSTEMATIC REVIEW

In order to associate the impact of dietary flavonoids on health outcomes, accurate measures of dietary intake are fundamental. The gaps in knowledge surrounding how dietary flavonoid intake is measured, presented in the Introduction (Chapter 1) of this thesis, indicated the need for improved methods for measuring dietary flavonoid consumption. A systematic literature review was conducted to provide an evaluation of the various methods employed by current literature to measure flavonoid intake, and their associated limitations. The review presents findings showing the reliance of studies utilising flawed dietary assessment methods to measure dietary flavonoid intake. Additionally, the review concludes that despite several methods that measure biomarkers of flavonoid intake being developed and validated, few studies apply biomarkers as a measure of dietary flavonoid intake.

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KK, KC and YP designed the study. KK and VG contributed to the literature search and sorting process. KK produced summary tables and the first draft of the manuscript. All authors contributed to writing and editing the manuscript.

To assist clarity of formatting, Tables 2-2, 2-3 and 2-4 are provided at the end of this chapter

2

2.1 Introduction

While the association between flavonoid intake and health outcomes is promising, more robust clinical data is needed. However, in order to associate flavonoid consumption with positive health outcomes, the first and most fundamental step is to accurately estimate dietary flavonoid intake. Despite the first estimations of flavonoid intake on a population level being reported more than a decade ago, the methods currently employed have evident flaws (1). Dietary flavonoid intake is difficult to quantify, and consequently numerous methods have been developed for application in various settings. With the absence of a gold standard approach, the methods utilised include various techniques within the fields of both dietary assessment and biomarker analyses.

There are currently numerous methods used to measure dietary flavonoid intake. With no gold standard method for measuring flavonoid intake, it is unclear which method for measuring or estimating dietary flavonoid intake is most useful in intervention and empirical studies. The limitations associated with current methods hinder the interpretation of observational and experimental research related to dietary flavonoid intake and specific health outcomes. An evaluation and comparison of the tools to measure flavonoid intake in observational and experimental research is imperative to interpret current findings across the literature and to provide recommendations for methods to apply in future research. Flavonoids are unlike other known nutrients in foods, and therefore their complexity and variability needs to be carefully considered. Traditional methods of dietary assessment require a recall or documentation of food intake from a given time period in either a prospective or retrospective manner (2). To determine the nutrient composition of individual or group intakes, this dietary intake data is required to be converted to an estimated consumption of the nutrient of interest. This can be done two ways, either applying a flavonoid specific food composition database (FCDB) to the dietary intake data; or through the assessment of biomarkers of intake that can be detected in biological samples.

To improve methodological quality of future research, a clear understanding of appropriate methods for measuring flavonoid intake is required. The proposed systematic review aims to provide an overview of available strategies for measuring or estimating dietary flavonoid intake and to provide an important resource for researchers when developing future studies.

The overall purpose of this review is to summarise and synthesise the evidence about the methods used in all published literature to measure or estimate dietary flavonoid intake in adults. The following question guided the literature search:

> "What method did researchers apply to determine intake of dietary flavonoids, using a dietary assessment or biomarker method?"

2.2 Methods

This review is registered with the International Prospective Register of Systematic Reviews (PROSPERO) under the registration number #CRD42014015607.

2.2.1 Types of studies

This review included randomised, non-randomised food-based trials, crossover food-based trials, and analytical epidemiological study designs including prospective and retrospective cohort, case-control and cross-sectional studies. Studies were restricted to those published from January 2004 to November 2014 to ensure the literature was current and relevant. Only studies published in English language were considered for inclusion due to a lack of translation resources.

2.2.2 Types of participants

Adults aged 18 years or over.

2.2.3 Types of data

Dietary assessment methods (and the application of a flavonoid related FCDB) that were utilised to investigate flavonoid intake were examined. Additionally, biomarkers

including intact flavonoids or flavonoid metabolites, quantified in biological samples (urine, plasma and/or faecal water) which are used to measure intake of flavonoids in habitual diet or a flavonoid rich food were also reviewed

Flavonoid intake is defined as:

- Total flavonoid intake
- Intake of a single or multiple flavonoid subclass including
    - Flavonols
    - Flavanols (flavan-3-ols)
    - Flavones
    - Flavanones
    - Anthocyanins (anthocyanidins)

Estimation of isoflavone intake will be excluded from this analysis due to the specific biological activities of isoflavones and the fact that isoflavone intake is often excluded from studies that address total flavonoid intake. Additionally, the breadth of isoflavone research is large enough to justify a distinct systematic review of the methods used to estimate intake.

1. Valid dietary assessment method employed, where applicable, which is cross-referenced with food composition data for determining flavonoid content of dietary intake/food/food groups. Including:

- 24-hour recall
- food record/diary
- food frequency questionnaire
- diet history

2. Biomarkers (intact flavonoids or associated metabolites) measured in biological samples using one or a combination of the following methods, where reported:

- chromatography (high performance liquid; gas; column; thin layer; paper)
- mass spectrometry;
- nuclear-magnetic resonance spectroscopy;

- Folin–Ciocalteu assay

Validation and application assessment studies were also included. The exclusion criteria were animal studies, food analytical experiment studies, studies related to essential oils, mechanistic studies (bioavailability, pharmacokinetics or mechanistic feeding studies) and reviews and meta-analyses. Studies were also excluded if 'in house' or unpublished FCBDs not available to researchers were utilised. Lastly, studies utilising non-specific biomarkers to measure flavonoid intake (i.e. antioxidant activity or markers of inflammation) were excluded (Table 2-1).

2.2.4 Search Strategy

The search aimed to find both published and unpublished studies through electronic databases, the World Wide Web and reference lists. Key terms were established:

- Phenolic acid
- Flavonoid*
- Flavanol*
- flavan-3-ol*
- Anthocyanin*
- Flavanone*
- Flavonol*
- Flavone*
- Polyphenol*

Table 2-1 Overall inclusion and exclusion criteria for screening

		Include if:
Language	Article published in English language	Yes
Design	Randomised or non-randomised food-based trials, or prospective or retrospective cohort or, case-control, cross-sectional studies. Case reports, reviews, editorials, letter to the editor, qualitative research	Yes No

Population	Adults aged 18+ years Animal studies or studies including <18 years	Yes No
Content	Studies examining total or specific dietary flavonoid intake through valid dietary assessment or biomarkers Pharmacokinetics/bioavailability studies of intake	Yes No
Access	Full-text article accessible	Yes

A three-step search strategy was utilised in this review. The initial phase consisted of searches of the following databases using the key terms searching in titles and abstracts in the following databases:

- Web of Science
- Scopus

A second stage includes searching for key terms in the following sites on www.googlescholar.com. A third stage of hand searching reference lists of articles retrieved to identify further studies of interest was conducted.

2.2.5 Selection of studies

One review author (YP) conducted the literature search in the specified scientific databases. Two review authors (KK and VG) independently assessed potential studies identified by the search strategy for inclusion. Resolution of any disagreements was mediated through discussion and as required, consultation with a third reviewer (YP) for consensus.

Results of the search were collated in the reference manager, Endnote (EndNote X4 for Windows, Thomson Reuters). Duplicate publications were excluded. Articles identified by database searches were assessed for relevance to the review based on the title and abstract (Table 2-1). For those meeting the inclusion criteria, the full text publication were retrieved and assessed for relevance to the review criteria.

2.2.6 Data extraction, management and synthesis

Relevant data relating to flavonoid sub group, study design, tool used and where relevant, the key methodological conclusions of the authors was extracted from the studies into an Excel template, prepared for the purpose of this review. The studies were grouped by total flavonoid intake and each flavonoid sub-group, described and evaluated in accordance to their methodological similarities. The findings are presented both in a narrative synthesis, as well as in tables and figures. When information regarding any of the above was unclear, attempt was made to contact authors of the original publication to provide further details.

2.3 Results

A PRISMA flow chart outlining the results of the search strategy and selection process was developed (Figure 2-1). The search returned 833 articles, after duplicates were removed, with 58 other potential studies identified through hand-sorting of reference lists. After the abstracts were screened, 174 potential studies were identified. A further 77 were excluded according to the inclusion/exclusion criteria, leaving 97 final papers in the review, with 76 estimating flavonoid intake using dietary assessment, and 20 relating to flavonoid biomarker analysis (including 15 validation studies and 5 application studies) (Figure 2-1).

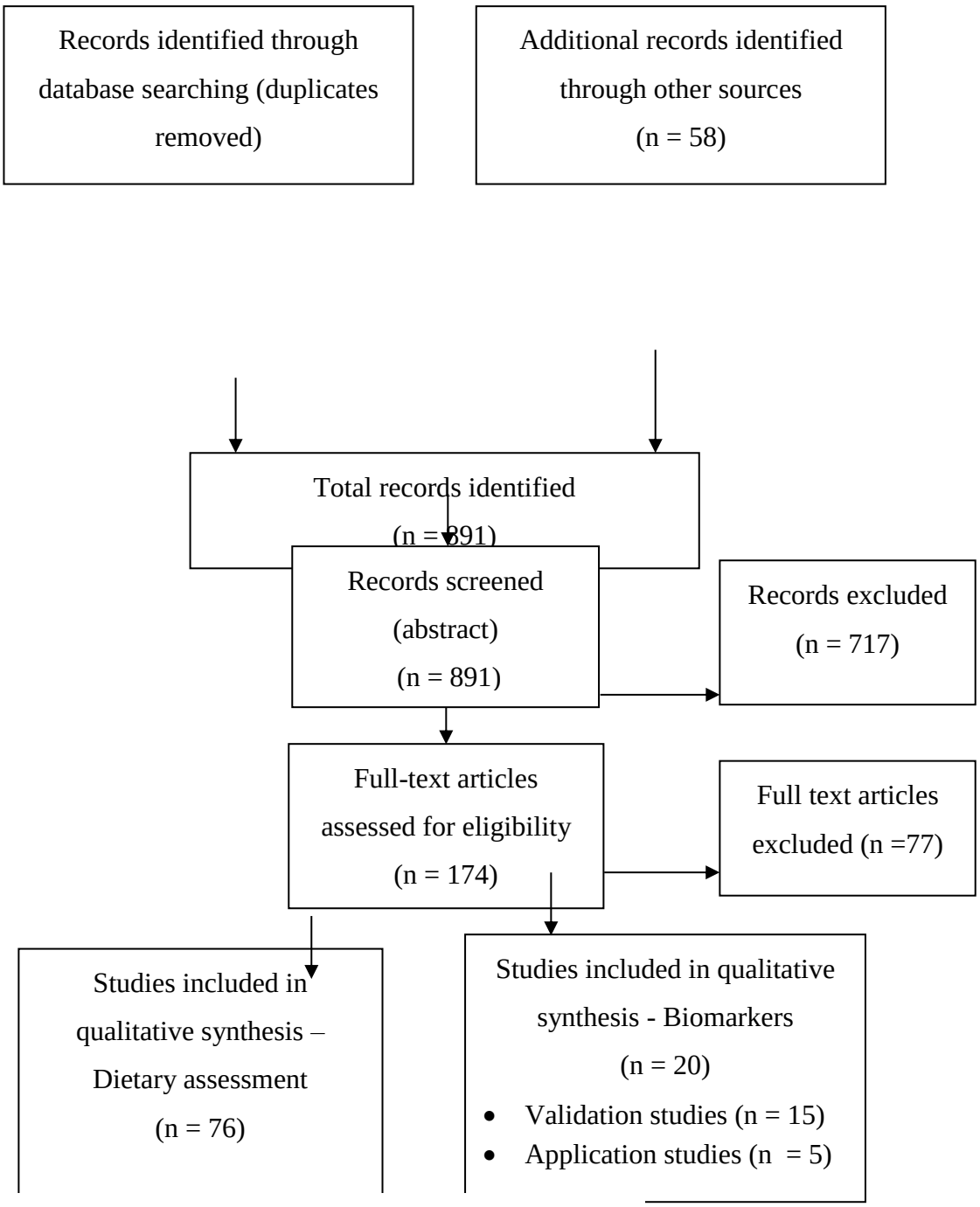

Figure 2-1 PRISMA flowchart of the search strategy

2.3.1 Estimation of flavonoid intake from dietary assessment (Table 2-2)

Details regarding the studies that estimate dietary flavonoid intake using dietary assessment are reported in Table 2-2. The study sample sizes ranged from 19 - 477,312 adults. The studies were conducted in the USA (n=28), Europe (multiple centre

studies) (n=9), Finland (n=6), Spain (n=4), China (n=2), the Netherlands (n=2), UK (n=2), Scotland (n=2), Australia (n=2), Mexico (n=2) and elsewhere. Study designs included cohort studies (n=39), cross-sectional analysis (n=18), case-control studies (n=16), randomised controlled trials (n=2) and an analytical study design (n=1).

Approximately a quarter of studies (n=19) were conducted with the primary aim of estimating flavonoid intake, or intake of flavonoid subclasses in populations using a dietary assessment tool. The majority of studies (n=57) estimated intakes to investigate the association of flavonoid consumption with health outcomes, including various cancers (n=29), cardiovascular diseases (n=14), diabetes (n=6), neuro-degenerative conditions (n=3) and others (n=5). The majority of studies utilised a FFQ to estimate dietary intake of flavonoids (n=57), while fewer estimated intake from 24 or 48h recalls (single or multiple) (n=12), diet history methods (n=5) and food records (n=2).

The reference FCDBs utilised in the studies included data from one or a combination of the USDA flavonoid database (2003, 2007, 2011) (n=49), the Phenol Explorer database (n=11), other published data sources (n=21) and other databases (n=5) (Euro FIR-eBASIS, 'functional food database').

Total flavonoid intake was reported by 58 studies (n=18 reported intake from a single or multiple subclass only). However, the culmination of subclasses and sum of individual flavonoids that contributed to this figure varied widely for each study, and differences mainly reflected the different FCDBs applied in each study (Table 2-2).

Most studies provided detailed information pertaining to the individual flavonoids that contributed to each subclass (the sum of which resulted in a measure of total flavonoid intake). 24 studies provided insufficient detail on the individual flavonoids which contributed to each subclass (denoted with NS – Table 2-2).

2.3.2 Estimation of flavonoid intake from biomarkers (Table 2-3)

Five studies were identified that utilised a biomarker as a measure of flavonoid intake as outlined in Table 2-3. These studies were case-controls (n=4) and cross sectional

(n=1) in nature, with populations ranging from 589 – 24,226 adults. Three studies measured biomarkers in urine and the other studies assessed biomarkers in plasma (n=2). Two studies assessed tea related biomarkers (EGC, EC, EGCG and ECG) using HPLC, one study measured quercetin, kaempferol, two studies measured EC, EGC and related metabolites using HPLC ESI-MS, and one study measured total polyphenols in urine using the Folin-Cicocalteu assay.

2.3.3 Biomarker validation studies (Table 2-4)

Fifteen studies were identified that validated methods developed to measure biomarkers of flavonoid intake or the intake of flavonoid-rich foods (Table 2-4). The study sample sizes ranged from 19 – 786 adults, and were conducted in Norway (n=2), Denmark (n=4), Australia (n=1), Africa (n=1), France (n=2), Spain (n=1), Finland (n=1), China (n=1) and the UK (n=2). Twelve studies reported utilising Liquid Chromatography, two studies reported utilising Gas Chromatography, and one study utilised the Folin–Ciocalteu assay. Ten studies reported measuring intact flavonoids in either urine or plasma. Other studies measured metabolites of flavonoid intake, including phenolic acids (n=1) or a combination of both (n=3). Four studies investigated the validity of the biomarkers in relation to total dietary flavonoid intake (n=2), or the intake of specific flavonoid subclasses (n=2). Eleven studies validated the biomarkers against the intake of flavonoid rich foods, such as fruit and vegetable intake, tea consumption or the intake of specific foods (citrus and berries). The reference methods were FFQs (n=7), food records (n=2), or controlled intervention diets (n=3), food recall (n=1), and two studies did not adequately report on the reference method utilised.

2.4 Discussion

This systematic literature review has presented and described the methods utilised by researchers to measure or estimate dietary flavonoid intake, and/or intake of flavonoid subclasses in studies, using a dietary assessment or biomarker method, published between 2004 and 2014.

The most common method of estimating flavonoid intake relied on dietary assessment of habitual food intake. Generally, usual dietary analysis was performed using a FFQ, a single or repeated 24-hour diet recall or a food record, which were cross-referenced with a flavonoid FCBD. This review highlighted that there are very few flavonoid specific FCBDs that exist globally and country-specific food composition data was rarely available. Aside from the limitations associated with each dietary assessment method (2), there are several well documented problems associated with utilising FCBDs to assign flavonoid content to selected foods, resulting in large variations in estimates of intake (3).

Firstly, estimation of dietary flavonoid intake is only as comprehensive as the composition database utilised. If, for example, a composition database does not have an extensive list of foods and the flavonoid content of a food in an individual's diet cannot be assigned or matched to its closest equivalent, then an individual's intake will certainly be under-estimated. This is particularly challenging when analysing food intake data from a country that does not have a specific composition database for that population, potentially resulting in significant sources of flavonoids for the population not being captured. The primary databases utilised by studies identified in this review (USDA, Phenol Explorer and Euro FIR-eBASIS) provide extensive and precise information on the individual flavonoids in foods, with additional detail provided on the variability of flavonoid contents in foods, as well as the values associated with processing circumstances. It is interesting to note that the utilisation of different databases may substantially influence the estimation of flavonoid intake in a study, as highlighted by a recent comparison between estimates of dietary flavonoid intake produced by the USDA and Phenol Explorer databases (4). However, this is not a well researched concept and needs further verification.

Secondly, the flavonoid content of specific foods is highly variable and largely influenced by a foods growth and processing conditions (5). Disregarding the processes by which foods are manufactured and the impact on flavonoid levels leads to an over-estimation of flavonoid intake. A flavonoid FCBD is largely unable to account for this variability and can only provide an estimate for each food consumed

(5). Studies did not generally apply retention factors, or comment if any methods were applied to manage flavonoid degradation associated with cooking, for instance.

Lastly, estimating dietary flavonoid intake through dietary assessment is unable to account for the high intra-individual variation associated with flavonoid metabolism and absorption, which is influenced by factors other than intake, such as bioavailability and genetic factors (6). Advances in informatics are crucial (5). Until the exact bioavailability of all flavonoids are understood and the individual variations in metabolism are accounted for, estimations of flavonoid intake and their correlation with health outcomes should be interpreted with caution.

Despite the validation of several tools developed to specifically measure flavonoid intake (7, 8), it appears that these tools have not been applied in further research. The relative validity of these tools for the measurement of flavonoid intake can be partially attributed to the fact that they effectively measure what they are hypothesised to measure (i.e. estimated dietary flavonoid exposure). However, as true flavonoid intake is difficult to establish, their objective validation, for example against a gold standard assessment, remains questionable. This review highlighted that studies tended to estimate flavonoid intake from a dietary assessment method that was not specifically intended for the purpose of measuring flavonoid intake (e.g., a general FFQ designed to capture energy intake). This can lead to misestimating exposure assessment, as foods included in the surveys may not be comprehensive enough or specific enough to capture the unique dietary exposure to flavonoids in that population. A major limitation of FFQs in this sense is the practice of combining foods that are similar nutritionally (e.g. apples and pears) into one question (thus, saving time coding and entering data). However, as the flavonoid content of foods is very specific, this can lead to misrepresentation of flavonoid intakes. It can not always be possible to collect detailed dietary information due to the very large numbers of participants in large epidemiological studies. However, the ability of these FFQs to capture flavonoid intake and the intake of flavonoid subclasses must be questioned and considered when interpreting the results of large studies.

While dietary flavonoid intake can be determined by quantifying biomarkers which include intact flavonoids and their metabolites found in plasma, urine and faecal water, there is an obvious lack of validated biomarkers of dietary flavonoid intake. While several of the validation studies outlined by this review (Table 2-4) found good correlation between specific biomarkers and the intakes of tea, fruits and vegetables (collectively), there is a lack of research to highlight the ability of a single, or multiple biomarkers as a measure of total dietary flavonoid intake, or even intake of flavonoid subclasses. This may be attributed to the difficulty in identifying useful biomarkers of flavonoid intake. Many thousands of flavonoids have been identified and after consumption they are quickly and extensively metabolised into various metabolites (9). Consequently, there are thousands of potential biomarkers (this is comprehensively explained in chapter 4 of this thesis) and there is no consensus around which flavonoids or metabolites are indicative of total dietary intake or reflective of intake for a certain food and/or food group. While many methods of measuring flavonoid biomarkers in human biological samples exist, it appears there is no standardised protocol of how to perform this analysis. This factor limits the comparison of studies that have used different methods to measure certain biomarkers.

The usefulness of urinary metabolites to measure polyphenol (including flavonoid intake) has been reviewed (10), and information pertaining to the bioavailability of certain biomarkers after intake of foods. However, the review did not include synthesis of studies that evaluated the ability of these biomarkers to measure dietary flavonoid intakes in population settings. Another review recently summarised biomarkers of fruit and vegetable intake applied in intervention studies (11). After reviewing 96 studies, the authors concluded that studies generally measured an antioxidant nutrient (carotenoid or vitamin C) and highlighted that relatively fewer studies measured flavonoid specific biomarkers such as quercetin (a flavonol). Some studies tried to measure total flavonoid excretion in urine, but no studies measured flavonoid biomarkers in plasma or faecal water.

Lastly, it is currently unknown which biological sample should be selected and some research suggests each may be indicative of different consumption patterns. Previous research has shown that urinary biomarkers of the flavonoid subclass of flavonoids

may be more reflective of short-term intake (12, 13). Additionally, biomarkers in fasting plasma or faecal water samples seem to be a suitable biomarker of short-term intake and a possible biomarker of the medium-term intake (12). However, biomarkers of long-term intake are not yet identified and may be unlikely due to the short half-lives of dietary flavonoids. Lastly, most of the biomarker analyses are expensive and often cannot be performed as part of large epidemiological studies (14). Future research needs to focus on identifying specific biomarkers of flavonoid intake and confirm the best methods in which to quantify these biomarkers in biological specimens.

2.4.1 Conclusion

This review has highlighted the reliance of studies measuring dietary flavonoid intake using dietary assessment methods (FFQs), with only a few studies associating biomarkers of flavonoid intake with health outcomes (15, 16). This review has also highlighted the considerable limitations surrounding the estimation of dietary flavonoid intake, which are unlikely to be overcome in the near future. However, the recent rapid advances in the understanding of flavonoid metabolism and bioavailability is a promising sign for continuing future developments. Future research needs to apply flavonoid specific dietary assessment methods that can adequately measure total flavonoid intake and the intake of specific subclasses. Additionally, future research needs to focus on identifying suitable biomarkers of flavonoid intake for an objective measurement of flavonoid intake. Until such biomarkers are available, reliance on dietary assessment methods will remain despite their many limitations.

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2.5 Tables 2-2 – 2-4

Table 2-2 Dietary assessment of flavonoid intake in published literature

Author	Study design	Method	Flavonoid total	Flavonoid Sub-classes						FCDB used	Population & Setting	Health outcome
				Flavonol	*Flavan-3-ol*	*Flavanone*	*Flavone*	*Anthocyanin*	*Other*			
Adebamowo et al., (24)	Cohort	FFQ, semi-quantitative	-	quercetin, kaempferol, myricetin	-	-	-	-	-	Published data (25) private food composition data	90,630 women, USA	Cancer, breast
Arem et al., (26)	Cohort	FFQ, semi-quantitative	Sum of sub-classes	NS	NS	NS	NS	NS	-	2007 USDA flavonoid database,	537 104 adults, USA	Cancer, pancreatic
Bai et al., (27)	Cross-sectional	24h recall	Sum of sub-classes	quercetin, kaempferol, myricetin, isorhamnetin	catechin, EGC, EC, EGCG, ECG, GC, CG TF, TDG, TFG	hesperetin, naringenin, eriodictyol	luteolin, apigenin	cyanidin, delphinidin, malvidin, pelargonidin, peonidin, petunidin	proanthocyanidins	2011 USDA flavonoid database	17 900 adults, USA	-
Bobe et al., (28)	RCT	FFQ, semi-quantitative	Sum of sub-classes	isorhamnetin, kaempferol, myricetin, quercetin	catechin, EGC, EC, EGCG, ECG, GC, CG TF, TDG, TFG	hesperitin, naringenin	luteolin, apigenin	cyanidin, delphinidin, malvidin, pelargonidin, peonidin, petunidin	-	2007 USDA flavonoid database	1,859 adults, USA	Cancer, colorectal

Bobe et al., (29)	Cohort	FFQ, quantitative	Sum of sub-classes	kaempferol, myricetin, quercetin	catechin, EC	-	luteolin, apigenin	-	-	Published data (25, 30-32)	27,111 adults, Finland	Cancer, pancreatic
Bosetti et al., (33)	Case control	FFQ	Sum of sub-classes	quercetin, myricetin kaempferol	EC, catechin	hesperitin narigerin	luteolin, apigenin	cyanidin malvidin	isoflavones	2003 USDA flavonoid database	5,157 adults, Italy	Cancer, breast
Butchart et al., (34)	Cross-sectional	FFQ, semi-quantitative	-	NS	catechins	NS	NS	-	procyanidins	a UK flavonoid database (35)	1091 adults, UK	Cognition
Cassidy et al., (36)	Cohort	FFQ, semi-quantitative	Sum of sub-classes	quercetin, kaempferol, myricetin, isohamnetin	catechins, EC	eriodictyol, hesperetin, naringenin	luteolin, apigenin	cyanidin, delphinidin, malvidin, pelargonidin, petunidin, peonidin	proanthocyanidins, theaflavins, thearubigins	2007 USDA flavonoid database, EuroFIR eBASIS	93 600 women, USA	Myocardial Infarction
Cassidy et al., (37)	Cohort	FFQ, semi-quantitative	Sum of sub-classes	quercetin, kaempferol, myricetin, isohamnetin	catechins, EC	eriodictyol, hesperetin, naringenin	luteolin, apigenin	cyanidin, delphinidin, malvidin, pelargonidin, petunidin, peonidin	proanthocyanidins, theaflavins, thearubigins	2007 USDA flavonoid database, EuroFIR eBASIS	87,242 women, USA	Hypertension
Cassidy et al., (38)	Cohort	FFQ, semi-quantitative	Sum of sub-classes	quercetin, kaempferol, myricetin, isohamnetin	catechins, EC	eriodictyol, hesperetin, naringenin	luteolin, apigenin	cyanidin, delphinidin, malvidin, pelargonidin, petunidin, peonidin	proanthocyanidins, theaflavins, thearubigins	2007 USDA flavonoid database, EuroFIR eBASIS	69 622 women, USA	Stroke
Chun et al., (39)	Cross-sectional	24h recall (single)	Sum of sub-classes	quercetin, kaempferol, myricetin, isohamnetin	catechins, EC, TF, TR	eriodictyol, hesperetin, naringenin	luteolin, apigenin	cyanidin, delphinidin, malvidin, pelargonidin,	isoflavones	2003 USDA flavonoid database	8809 adults, USA	-

								peonidin, petunidin				
Chun et al., (40)	Cross-sectional	24h recall (single)	Sum of sub-classes	quercetin, kaempferol, myricetin, isohamnetin	catechins, EC, TR, TF	eriodictyol, hesperetin, naringenin	luteolin, apigenin	cyanidin, delphinidin, malvidin, pelargonidin, petunidin, peonidin	isoflavones	2003 USDA flavonoid database	8809 adults, USA	-
Cui et al., (41)	Case-control	FFQ, semi-quantitative	Sum of sub-classes	quercetin, kaempferol, myricetin, isohamnetin	catechins, EC, TR, TF	eriodictyol, hesperetin, naringenin	luteolin, apigenin	cyanidin, delphinidin, malvidin, pelargonidin, petunidin, peonidin	-	2007 USDA flavonoid database	1395 adults, USA	Cancer, lung
Cutler et al., (42)	Cohort	FFQ, semi-quantitative	Sum of sub-classes	NS	NS	NS	NS	NS	isoflavones, proanthocyanidins	2003 USDA flavonoid database	34,708 women, USA	Cancer, lung, colorectal, breast, pancreatic and upper digestive cancer
Dilis et al., (43)	Cohort	FFQ, semi-quantitative	Sum of sub-classes	quercetin, kaempferol, myricetin, isohamnetin	catechin, EGC, EC, EGCG, ECG, GC, CG	eriodictyol, hesperetin, naringenin	luteolin, apigenin	cyanidin, delphinidin, malvidin, pelargonidin, petunidin, peonidin	Proanthocyanidins, TAC indexes,	2007 USDA flavonoid database	28572 adults, Greece	-
Djuric et al., (44)	Case-control	FFQ, semi-quantitative	-	quercetin	-	-	-	-	-	NS	2664 adults, USA	Cancer, colorectal
Dwyer et al., (45)	RCT	24h recall (x3)	Sum of sub-classes	quercetin, kaempferol, myricetin, isorhamnetin	catechin, EGC, EC, EGCG, ECG, GC,	eriodictyol, hesperetin, naringenin	luteolin, apigenin	cyanidin, delphinidin, malvidin, pelargonidin,	isoflavones	2003 USDA flavonoid database	550 women, USA	-

					CG TF, TDG, TFG			petunidin, peonidin				
Fink et al., (46)	Cohort	FFQ	Sum of sub-classes	NS	NS	NS	NS	NS	isoflavones, lignans	2003 USDA flavonoid database	1210 women, USA	Cancer, breast survival
Fisher et al., (47)	Analytical study	FFQ	NS	-	-	-	-	-	proanthocyanidin	2003 USDA flavonoid database	19 older adults, USA	Endothelial function
Galvan-Portillo et al., (48)	Cohort	FFQ, semi-quantitative	Sum of sub-classes	NS	NS	-	NS	-	-	2003 USDA flavonoid database	50 women, Mexico	-
Gates et al., (49)	Cohort	FFQ, semi-quantitative	Sum of sub-classes	myricetin, kaempferol, quercetin	-	-	Luteolin, apigenin	-		Published (25) and unpublished data	66,940 women, USA	Cancer, ovarian
Gates et al., (50)	Case-control	FFQ, semi-quantitative	Sum of sub-classes	myricetin, kaempferol, quercetin	-	-	Luteolin, apigenin	-	-	2007 USDA flavonoid database	2324 women, USA	Cancer, ovarian
Geybels et al., (51)	Case-control	FFQ	Sum of sub-classes	myricetin, kaempferol, quercetin	catechin, EC	-	-	-	-	Published data (25, 31, 52, 53)	58,279 men, Netherlands	Cancer, prostate
González et al., (54)	Cross-sectional	FFQ, semi-quantitative	Sum of sub-classes	NS	NS	NS	NS	NS	phenolic acids, lignans, stilbenes	Phenol-Explorer database	159 older adults, Spain	Lipid oxidative damage
Grosso et al., (55)	Cross-sectional	FFQ, semi-quantitative	Sum of sub-classes	NS	NS	NS	NS	NS	isoflavones, phenolic acids, lignans,	Phenol-Explorer database	10477 adults, Poland	-

									stilbenes, alkylphenols, tyrosols			
Hardcastel et al., (56)	Cross-sectional	FFQ	Sum of sub-classes	quercetin	Catechin, EC	Hesperitin, narigenin	NS	-	isoflavones, proanthocyanidins	Published data (35) and 2003 USDA flavonoid database	3226 women, Scotland	Bone health
Hernandez-Ramirez et al., (57)	Case-control	FFQ, semi-quantitative	Sum of sub-classes	NS	NS	-	NS	-	phenolic acids, lignans, coumestans	2003 USDA flavonoid database	735 adults, Mexico	Cancer, gastric
Hirvonen et al., (58)	Cohort	Self-reported, modified diet history method	-	NS	-	-	NS	-	-	Published data (25, 31, 32)	25,041 men, Finland	Intermittent claudication
Hirvonen et al.,(59)	Cohort	Self-reported, modified diet history method	-	NS	-	-	NS	-	-	Published data (25, 31, 32)	27,110 men, Finland	Cancer
Hughs et al., (60)	Cohort	FFQ, semi-quantitative	-	myricetin, kaempferol, quercetin	catechin, EGC, EC, EGCG, ECG, GC, CG	-	-	-	-	Published data (25, 31, 52, 53)	4280 adults, Netherlands	Stroke risk
Ivey et al., (61)	Cohort	FFQ, semi-quantitative	Sum of sub-classes	quercetin, kaempferol, myricetin, isohamnetin	catechin, EGC, EC, EGCG, ECG, GC, CG TF,	eriodictyol, hesperetin, naringenin	luteolin, apigenin	cyanidin, delphinidin, malvidin, pelargonidin, petunidin, peonidin	proanthocyanidins, isoflavones	2007 USDA flavonoid database	1063 older women, Australia	Atherosclerotic vascular disease

					TDG, TFG							
Jacques et al., (62)	Cohort	FFQ, semi-quantitative	Sum of sub-classes	Quercetin, kaempferol, myricetin, isohamnetin	catechin, EGC, EC, EGCG, ECG, GC, CG	Eriodictyol, hesperetin, naringenin	luteolin, apigenin	Cyanidin, delphinidin, nnalvidin, pelargonidin, petunidin, peonidin	proanthocyanidin	2007 USDA flavonoid database	2915 adults, USA	Diabetes
Knaze et al., (63)	Cohort	24 recall	-	-	catechin, EGC, EC, EGCG, ECG, GC, CG TF, TDG, TFG	-	-	-	proanthocyanidin	2009 Phenol-Explorer database, 2007 USDA flavonoid database	36 037 adults, Europe	-
Lagiou et al., (64)	Case-control	FFQ, semi-quantitative	Sum of sub-classes	NS	NS	NS	NS	NS	isoflavones	2003 USDA flavonoid database	200 adults, Greece	Peripheral arterial occlusive disease
Lagiou et al., (65)	Case-control	FFQ, semi-quantitative	Sum of sub-classes	NS	NS	NS	NS	NS	isoflavones	2003 USDA flavonoid database	899 adults, Greece	Coronary heart diseases
Laurin et al., (66)	Cohort	24h recall	NS	-	-	-	-	-	-	Published data (31)	2,459 men, Honolulu-Asia	Dementia
Letenneur et al., (67)	Cohort	FFQ	Sum of sub-classes	quercetin, kaempferol, myricetin	-	-	luteolin, apigenin	-	-	Published data (25)	1064 older adults, France	Cognitive decline
Lin et al., (68)	Cohort	FFQ, semi-quantitative	-	quercetin, kaempferol, myricetin	-	-	luteolin, apigenin	-	-	Published data (25, 69, 70)	66,360 women, a, USA	Coronary heart disease

McCullough et al., (71)	Cohort	FFQ, semi-quantitative	Sum of sub-classes	quercetin, kaempferol, myricetin, isohamnetin	catechin, EGC, EC, EGCG, ECG, GC, CG	eriodictyol, hesperetin, naringenin	luteolin, apigenin.	cyanidin, delphinidin, malvidin, pelargonidin, petunidin, peonidin	proanthocyanidins, isoflavones	2007 USDA flavonoid database	98469 adults, USA	Cardiovascular disease mortality
Mink et al., (72)	Cohort	FFQ, semi-quantitative	Sum of sub-classes	quercetin, kaempferol, myricetin, isorhamnetin	catechin, EGC, EC, EGCG, ECG, GC, CG TF, TDG, TFG ECG,EGCG	eriodictyol, hesperetin, naringenin	luteolin, apigenin	cyanidin, delphinidin, malvidin, pelargonidin, petunidin, peonidin	proanthocyanidins, isoflavones	2003 USDA flavonoid database	34 489 women, USA	Cardiovascular disease mortality
Mullie et al., (73)	Cross-sectional	FFQ, semi-quantitative	Sum of sub-classes	quercetin, kaempferol, myricetin, isorhamnetin	catechin, EGC, EC, EGCG, ECG, GC, CG	eriodictyol, hesperetin, naringenin	luteolin, apigenin	cyanidin, delphinidin, malvidin, pelargonidin, petunidin, peonidin	proanthocyanidins, isoflavones	2003 USDA flavonoid database	45 women, Belgium	-
Mursu et al., (74)	Cohort	4-day FR	Sum of sub-classes	quercetin, kaempferol, myricetin, isorhamnetin	catechin, EGC, EC, EGCG, ECG, GC, CG	eriodictyol, hesperetin, naringenin	luteolin, apigenin	cyanidin, delphinidin, malvidin, pelargonidin, petunidin, peonidin	-	2003 USDA flavonoid database and published literature (75)	1380 men, Finland	Carotid atherosclerosis
Musru et al., (76)	Cohort	4-day FR	Sum of sub-classes	quercetin, kaempferol, myricetin, isorhamnetin	catechin, EGC, EC, EGCG, ECG, GC, CG	eriodictyol, hesperetin, naringenin	luteolin, apigenin	cyanidin, delphinidin, malvidin, pelargonidin,	-	2003 USDA flavonoid database and	2590 men, Finland	Cancer

								petunidin, peonidin		published literature (75)		
Nettleton et al., (77)	Cohort	FFQ	Sum of sub-classes	NS	NS	NS	NS	NS	proanthocyanidins, isoflavones	2003 USDA flavonoid database	35816 women, USA	Diabetes (Type II)
Nothlings et al., (78)	Cohort	FFQ, quantitative	-	quercetin, kaempferol, myricetin	-	-	-	-	-	Published literature (79)	424 978 adults, Europe	Cancer, pancreatic
Nothlings et al., (80)	Cohort	FFQ, semi-quantitative	-	quercetin, kaempferol, myricetin	-	-	-	-	-	Published literature (79)	183,518 adults, USA	Cancer, pancreatic
Ock et al., (81)	Cross-sectional	24h recall	Sum of sub-classes	quercetin, kaempferol, myricetin, isohamnetin	catechins, EC, TR, TF	eriodictyol, hesperetin, naringenin	luteolin, apigenin	cyanidin, delphinidin, malvidin, pelargonidin, petunidin, peonidin	isoflavones	2003 USDA flavonoid database	8809 adults, USA	-
Otaki et al., (82)	Cross-sectional	FFQ	Sum of sub-classes	quercetin, kaempferol, myricetin, fisetin	EC, EGC, catechin	Hesperitin, narigenin	luteolin, apigenin	-	isoflavones	Functional Food Factor database	569 women, Japan	-
Ovaskainen et al., (83)	Cross-sectional	48h recall	-	Isorhamnetin, kaempherol, myricetin, and quercetin	EC, catechin	Eriodictyol, hesperetin, and naringenin.	-	cyanidin, delphinidin	phenolic acids	Published data (25, 32)	2007 adults, Finland	-
Perez Jimenez et al., (84)	Cross-sectional	24h recall (x6)	-	NS	NS	-	NS	NS	phenolic acids, lignans, stilbenes, minor	Phenol-Explorer database	4942 adults, France	-

									polyphenols			
Rossi et al., (85)	Case-control	FFQ, semi-quantitative	Sum of sub-classes	NS	NS	NS	NS	NS	proanthocyanidins, isoflavones	2003 USDA flavonoid database	1362 women, Italy	Cancer, endometrial
Rossi et al., (86)	Case-control	FFQ, semi-quantitative	Sum of sub-classes	NS	NS	NS	NS	NS	proanthocyanidins, isoflavones	2003 USDA flavonoid database	978 adults, Italy	Cancer, pancreatic
Rossi et al., (87)	Case-control	FFQ, semi-quantitative	Sum of sub-classes	NS	NS	NS	NS	NS	isoflavones	2003 USDA flavonoid database	3442 women, Italy	Cancer, ovarian
Rossi et al., (88)	Case-control	FFQ, semi-quantitative	Sum of sub-classes	NS	NS	NS	NS	NS	isoflavones	2003 USDA flavonoid database	6107 adults, Italy	Cancer, colorectal
Somerset et al., (14)	Cross-sectional	FFQ, semi-quantitative	Sum of sub-classes	Sum of sub-classes	isorhamnetin, kaempferol, myricetin, quercetin	catechin, EGC, EC, EGCG, ECG, GC, CG TF, TDG, TFG	eriodictyol, hesperetin, naringenin	luteolin, apigenin	cyanidin, delphinidin, malvidin, pelargonidin, peonidin, petunidin	2007 USDA flavonoid database	60 adults, Australia	-
Simons et al., (89)	Cohort	FFQ, semi-quantitative	-	myricetin, kaempferol, quercetin	catechin, EGC, EC, EGCG, ECG, GC, CG	-	-	-	-	Published data (25, 31, 90)	120852 adults, Netherlands	Cancer, colorectal
Song et al., (91)	Cross-sectional	FFQ, semi-quantitative	-	myricetin, kaempferol, quercetin	-	-	luteolin, apigenin	-	-	Published data (25, 31)	38,018 women, USA	Diabetes (Type II)
Tavani et al., (92)	Case-control	FFQ, semi-quantitative	Sum of sub-classes	NS	NS	NS	NS	NS	isoflavones	2003 USDA	1442 adults, Italy	Myocardial infarction

										flavonoid database		
Theodoratou et al., (93)	Case-control	FFQ, semi-quantitative	-	quercetin	Catechin, EC	Hesperitin, narigenin	NS	-	isoflavones, proanthocyanidins	Published data (35)	2912 adults, Scotland	Cancer, colorectal
Wang et al., (94)	Cohort	FFQ, semi-quantitative	Sum of sub-classes	quercetin, kaempferol, myricetin	-	-	Luteolin, apigenin	-	-	Published data (25, 31, 70)	38,408 women, USA	Cancer
Wang et al., (95)	Cohort	FFQ, semi-quantitative	Sum of sub-classes	NS	NS	NS	NS	NS	isoflavones, proanthocyanidins	2007 USDA flavonoid database	43,268 men, USA	Prostate cancer
Wedick et al., (96)	Cohort	FFQ, semi-quantitative	Sum of sub-classes	NS	NS	NS	NS	cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and petunidin	-	2007 USDA flavonoid database	200,894 adults, USA	Diabetes (Type II)
Welch et al., (97)	Cross-sectional	FFQ, semi-quantitative	Sum of sub-classes	quercetin, kaempferol, myricetin, isohamnetin	catechins, epicatachins	eriodictyol, hesperetin, naringenin	luteolin, apigenin.	cyanidin, delphinidin, malvidin, pelargonidin, petunidin, peonidin	proanthocyanidins	2007 USDA flavonoid database	3160 women, UK	Bone mineral denisty
Woo et al., (98)	Case-control	FFQ, semi-quantitative	Sum of sub-classes	NS	NS	NS	NS	NS	isoflavones	Published data (99)	668 adults, Korea	Cancer, Gastric
Zamora-Ros et al., (100)	Cohort	FFQ or Diet history questionnaire	Sum of sub-classes	isorhamnetin, kaempferol, myricetin, quercetin	catechin, EGC, EC, EGCG, ECG, GC, CG TF, TDG, TFG	eriodictyol, hesperetin, naringenin	luteolin, apigenin	cyanidin, delphinidin, malvidin, pelargonidin, peonidin, petunidin	lignans, procyanidins, isoflavones	2009 Phenol-Explorer database, 2007 USDA	477,312 adults, Europe	Cancer, gastric

										flavonoid database		
Zamora-Ros et al., (101)	Cross-sectional	Diet history questionnaire	Sum of sub-classes	isorhamnetin, kaempferol, myricetin, quercetin	catechin, EGC, EC, EGCG, ECG, GC, CG TF, TDG, TFG	eriodictyol, hesperetin, naringenin	luteolin, apigenin	cyanidin, delphinidin, malvidin, pelargonidin, peonidin, petunidin	procyanidins, isoflavones	2007 USDA flavonoid database, UK Food Standards Agency	40,683, Spain	-
Zamora-Ros et al.,(102)	Cohort	FFQ or Diet history questionnaire	Sum of sub-classes	isorhamnetin, kaempferol, myricetin, quercetin	catechin, EGC, EC, EGCG, ECG, GC, CG TF, TDG, TFG	eriodictyol, hesperetin, naringenin	luteolin, apigenin	cyanidin, delphinidin, malvidin, pelargonidin, peonidin, petunidin	lignans, procyanidins, isoflavones	2009 Phenol-Explorer database, 2007 USDA flavonoid database	334,850 women, Europe	Cancer, breast
Zamora-Ros et al.,(103)	Cohort	FFQ or Diet history questionnaire	Sum of sub-classes	isorhamnetin, kaempferol, myricetin, quercetin	catechin, EGC, EC, EGCG, ECG, GC, CG TF, TDG, TFG	eriodictyol, hesperetin, naringenin	luteolin, apigenin	cyanidin, delphinidin, malvidin, pelargonidin, peonidin, petunidin	lignans, procyanidins, isoflavones	2009 Phenol-Explorer database, 2007 USDA flavonoid database	340,234 adults, Europe	Diabetes (Type II)
Zamora-Ros et al., (104)	Cohort	FFQ or Diet history questionnaire	-	isorhamnetin, kaempferol, myricetin, quercetin	catechin, EGC, EC, EGCG, ECG, GC, CG	-	-	-	proanthocyanidin	2009 Phenol-Explorer database, 2007 USDA flavonoid database	340,234 adults, Europe	Diabetes (Type II)

Zamora-Ros et al., (105)	Cohort	Diet history questionnaire	Sum of sub-classes	isorhamnetin, kaempferol, myricetin, quercetin	catechin, EGC, EC, EGCG, ECG, GC, CG TF, TDG, TFG	eriodictyol, hesperetin, naringenin	luteolin, apigenin	cyanidin, delphinidin, malvidin, pelargonidin, peonidin, petunidin	lignans, procyanidins, isoflavones	2009 Phenol-Explorer database, 2007 USDA flavonoid database,	40,622 adults, Spain	Mortality
Zamora-Ros et al., (106)	Cohort	24h recall	Sum of sub-classes	isorhamnetin, kaempferol, myricetin and quercetin	catechin, EGC, EC, EGCG, ECG, GC, CG	eriodictyol, hesperetin and naringenin	apigenin and luteolin,	cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin	isoflavones	2009 Phenol-Explorer database, 2007 USDA flavonoid database	35 628 adults, Europe	-
Zamora-Ros et al., (107)	Cohort	24h recall	-	isorhamnetin, kaempferol, myricetin and quercetin	-	eriodictyol, hesperetin and naringenin	apigenin and luteolin,	-	-	2007 USDA flavonoid database	36 037 adults, Europe	-
Zamora-Ros et al., (108)	Cohort	24h recall	-	-	-	-	-	cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin	-	2007 USDA flavonoid database	36 037 adults, Europe	-
Zamora-Ros et al., (109)	Case-control	Diet history questionnaire	Sum of sub-classes	isorhamnetin, kaempferol, myricetin, quercetin	catechin, EGC, EC, EGCG, ECG TF,	eriodictyol, hesperetin, naringenin	luteolin, apigenin	cyanidin, delphinidin, malvidin,	lignans, procyanidins, isoflavones	2009 Phenol-Explorer database, 2007	825 adults, Spain	Cancer, colorectal

					TDG, TFG			pelargonidin, peonidin, petunidin		USDA flavonoid database		
Zhang et al., (110)	Cross-sectional	FFQ, semi-quantitative	-	quercetin, myricetin, kaempferol, isorhamnetin	-	-	luteolin, apigenin	-	-	Published data (111)	5,046 adults, China	-
Zhang et al., (112)	Cross-sectional	FFQ, semi-quantitative	Sum of sub-classes	NS	NS	NS	NS	NS	proanthocyanidins	2007 USDA flavonoid database	3317 adults, China	Bone mineral density

NS=details of individual flavonoids not specified, - = no data provided, FCDB = food composition database

(-)-epigallocatechin (EGC), (-)-epicatechin (EC), (-)-epigallocatechin-3-gallate (EGCG), (-)-epicatechin-3-gallate (ECG), gallocatechin (GC), catechin3-gallate (CG)

theaflavin (TF), theaflavin 3,3-digallate (TDG), theaflavin 3-gallate (TFG), thearubigin (TR)

Table 2-3 Studies identified that utilised a biomarker as a measure of flavonoid intake

Author	Study design	Method	Biological sample	Biomarkers	Health outcome	Population & Setting
Iwasaki et al., (22)	Nested case-control	HPLC with colorimetric array detector	Plasma	EGC, EC, EGCG, ECG	Cancer, breast	24,226 women, Japan
Luo et al., (23)	Nested case-control	HPLC/ESI-MS	Urine	Quercetin, kaempferol, EC, EGC, M4, M6, 40-MeEGC	Cancer, breast	353 incident breast cancer cases, 701 individually matched controls, Japan
Medina-Remón et al., (113)	Cross-sectional	Folin-Ciocalteu assay	Urine (spot)	Total polyphenol	Blood pressure	589 adults, Spain
Sasazuki et al., (114)	Nested case-control	HPLC	Plasma	EGC, EC, EGCG, ECG	Cancer, gastric	494 gastric cancer cases, 494 Controls, Japan
Yuan et al., (115)	Nested-case-control	LC/ESI-MS, NMR	Urine	EGC, 40-MeEGC, EC, M4, M6	Cancer, colorectal	162 colorectal cancer cases, 806 controls, China

high-performance liquid chromatography (HPLC), liquid chromatography (LC), electrospray ionization (ESI), mass spectrometry (MS), nuclear magnetic resonance (NMR)

(-)-epigallocatechin (EGC), (-)-epicatechin (EC), (-)-epigallocatechin 3-gallate (EGCG), (-)-epicatechin 3-gallate (ECG),

5-(30, 40, 50-trihydroxyphenyl)-c-valerolactone (M4), 5-(30,40,-dihydroxyphenyl)-cvalerolactone (M6), 40-MeEGC, the methylated form of EGC,

Table 2-4 Validation of methods developed to measure biomarkers as markers of total flavonoid intake or intake of flavonoid rich foods

Author	Design	Reference method	Biomarker	Method	Biological Sample	Flavonoid sub-classes or dietary intake estimated	Population & Setting	Sig. methodological conclusions
Brantsæter et al., (116)	Cross-sectional	FFQ	hesperitin, naringenin, eriodictyol, phloretin, quercetin, kaempferol, isorhamnetin, tamarixetin apigenin	LC/MS	Urine	Fruit, vegetables, tea intake	119 pregnant women, West Africa	FFQ estimate of fruit intake was significantly correlated with phloretin (r = 0.33), citrus fruit/juice with hesperetin (r= 0.44), tea with kaempferol (r = 0.41) (P < 0.01 for all)
Bredsdorff et al., (117)	Nested case-control study	FFQ	hesperetin, naringenin, quercetin, kaempferol, isorhamnetin, tamarixetin, genistein, daidzein, apigenin, phloretin	HPLC-MS	Urine	Individual flavonoids, sum sub-classes, sum total	393 cases (acute coronary syndrome) 393 non-cases, Denmark	Correlations between urinary kaempferol excretion and intakes of tea 0.34 (P < 0.0001), wine 0.17 (P < 0.0001), vegetables 0.08 (P = 0.020)
Brevik et al., (118)	Randomised cross-over study	Controlled intervention diet	eriodictyol, naringenin, hesperetin; quercetin, kaempferol, isorhamnetin, tamarixetin, phloretin	HPLC-MS	Urine	Fruit, vegetable intake	40 adults, Norway	Increased intake of mixed fruits and vegetables from 2 to 5 servings/day significantly increased urinary excretion of eriodictyol, naringenin, hesperetin, quercetin, kaempferol, isorhamnetin, and tamarixetin.
Cao et al., (119)	Cross-sectional study	7-day food records	quercetin, kaempferol, isorhamnetin, apigenin, luteolin	HPLC	Plasma (fasting)	quercetin, kaempferol, isorhamnetin, apigenin, luteolin	92 adults, China	The mean 7 d intake of quercetin, kaempferol, isorhamnetin, apigenin, and luteolin was positively correlated to corresponding plasma concentrations, with correlation coefficients ranging from 0·33 to 0·51 (all P<0·05).
Carlsen et al., (120)	Cross-sectional	FFQ	apigenin, eriodictyol, hesperidin, kaempferol, naringenin, phloretin, quercetin, tamarixetin, isorhamnetin	HPLC	Urine (2x24h urine samples)	Fruit, vegetables, juice intake	85 adults, Norway	Total urinary flavonoids showed good correlation with fruit, juice, and vegetable intake. The highest correlations were observed between the

								intakes of fruit (including juice) with hesperidin and naringenin biomarkers.
Hodgson et al., (121)	Cross-sectional	Self reported tea intake (cups/ day)	4-O-methylgallic acid (4OMGA), isoferulic acid	GC-MS	Urine (24h)	Tea, coffee intake (# cups/day), no detail on data collection method	455 adults, Australia	4OMGA was related to usual (r 0·50, P<0·001) and current (r 0·57, P<0·001) tea intake, isoferulic acid was related to usual (r 0·26, P=0·008) current (r 0·18, P<0·001) coffee intake.
Huxley et al., (122)	Randomised-controlled trial	FFQ	quercetin, kaempferol, isorhamnetin	Not reported	Plasma	Fruit, vegetable intake	690 adults, UK	No change in total non fasting plasma flavonol concentrations between baseline and follow up in either the control or intervention group. Flavonol measurements may not predict total fruit, vegetable intake.
Koli et al., (123)	Randomised, placebo-controlled dietary intervention trial.	Controlled intervention diet	quercetin, p-coumaric acid, 3-hydroxyphenylacetic acid, caffeic acid, protocatechuic acid, vanillic acid, homovanillic acid, 3-(3-hydroxyphenyl)propionic acid	GC-MS, HPLC	Plasma (fasting), Urine (24h)	Berry intake	72 adults, Finl	Plasma quercetin, *p*-coumaric acid, 3-hydroxyphenylacetic acid, caffeic acid, protocatechuic acid, vanillic acid, homovanillic acid, 3-(3-hydroxyphenyl)propionic acid increased significantly from the baseline in the berry group compared to the control group ($p < 0.05$). Urinary excretion of quercetin, *p*-coumaric acid, 3-hydroxyphenylacetic acid increased significantly in the berry group compared to the control group ($p < 0.05$). All the biomarkers may reflect a diet containing moderate amounts of blue/ red berries.
Krogholm et al., (124)	Cross-sectional	24h recall	quercetin, isorhamnetin, tamarixetin, kaempferol, hesperetin, naringenin, eriodictyol, daidzein, genistein, phloretin	HPLC-EI-MS	Urine (24h)	Fruit intake	103 adults, Denmark	24 h urinary excretion of total flavonoids and the estimated intake of fruits were correlated (r0.31, P0.01). Intake of citrus fruits, citrus juices was correlated with total excretion of citrus specific flavonoids (r0.28, P0.01), orange was

								positively correlated with naringenin (r0.24, P0.01) hesperetin (r0.24, P0.01). Phloretin was correlated with apple intake (r0.22, P0.01) and overall estimated intake of fruit (r0.22, P0.01).
Krogholm et al., (125)	Cross-sectional	FFQ	quercetin, isorhamnetin, tamarixetin, kaempferol, hesperetin, naringenin, eriodictyol, phloretin apigenin	HPLC-EI -MS	Urine (spot, morning void)	Fruit, vegetables, beverages	191 adults, Denmark	The intakes of fruit, juice, vegetables and tea reported in the FFQ were reflected by the flavonoid biomarker both in overnight and 24h urine samples. Validity coefficients for the flavonoid biomarker in overnight urine ranged from 0.39 to 0.49, while the corresponding validity coefficients for the biomarker in 24h urine ranged from 0.43 to 0.66.
Krogholm et al., (126)	Randomised, controlled crossover study	Intervention diet	flavonols quercetin, kaempferol, isorhamnetin, tamarixetin, the flavanones hesperetin naringenin, the dihydrochalcone, phloretin	HPLC-EI-MS	Urine (spot, morning void) vs Urine (24h)	Fruit, vegetable intake	12 men, Denmark	The total excretion of flavonoids in 24-h urine samples increased linearly with increasing fruit vegetable intakes (r_s = 0.86, P < 0.001). The total excretion of flavonoids in morning urine also increased, but the association was weaker (r = 0.59, P < 0.0001)
Medina-Remo´n et al., (113)	Cross-sectional	FFQ	Total polyphenol	Folin-Ciocalteu assay vs FFQ	Urine (spot)	Total polyphenol intake	589 adults, Spain	The intake of 100 g of F&V (β=0.150;P < 0.001) had a greater contribution to TPE than 100 mL of coffee β=0.141;P=0.001), the latter two foods contributed more than the consumption of 100 mL of wine (β=0.120;P=0.019).
Mennen et al., (20)	Cross-sectional	2-day FR	chlorogenic acid, caffeic acid, m-coumaric acid, gallic acid, 4-O-methylgallic acid, quercetin,	HPLC–EI–MS	Urine (24h) vs urine (spot)	Polyphenol rich food intake	53 adults, France	Spot urine: apple intake positively correlated to phloretin, grapefruit intake to naringenin, orange to hesperetin, citrus fruit consumption to both naringenin and hesperitin (r

			isorhamnetin, kaempferol, hesperetin, naringenin, phloretin, enterolactone enterodiol,					between 0·31 to 0·57) (P<0·05) All fruits, juices positively correlated to gallic acid, 4-O-methylgallic acid, isorhamnetin, kaempferol, hesperetin, naringenin, phloretin (r 0·24–0·44, P<0·05). Coffee consumption positively correlated to caffeic, chlorogenic acids (r 0·29 0·63, P<0·05 respectively). Black tea, wine consumption were positively correlated with gallic, 4-O-methylgallic acids (r 0·37–0·54, P<0·001). The present results suggest that several polyphenols measured in a spot urine sample can be used as biomarkers of polyphenol-rich food intake.
Pujos-Guillot (127)	Cohort study	NS	Flavanone glycosides	LC-MS	Urine (spot)	Citrus intake	80 adults (40 low 40 high citrus consumers), France	Hesperetin 3′-O-glucuronide, naringenin 7-O-glucuronide were identified as potential biomarkers of citrus intake, however the assessment was not sensitive enough to discriminate high low citrus consumers
Ranka et al., (128)	Cross-sectional	FFQ	quercetin and naringenin	HPLC	Urine (24h)	quercetin and naringenin intake	49 adults, UK	The correlation between FFQ estimated intake of quercetin and naringenin and levels excreted in the urine were r0.82 (P<0.0001) and r0.25 (P=0.05), respectively.

high-performance liquid chromatography (HPLC), liquid chromatography (LC), electrospray ionization (ESI), mass spectrometry (MS)

food frequency questionnaire (FFQ), food record (FR), food composition database (FCDB)

NS- not specified

CHAPTER 3: A SECONDARY DATA ANALYSIS CONDUCTED TO INFORM THE DEVELOPMENT AND VALIDATION OF A FOOD FREQUENCY QUESTIONNAIRE TO MEASURE FLAVONOID INTAKE IN OLDER AUSTRALIANS

Accurate estimation of the impact of dietary flavonoids on health outcomes is dependent on the tools used to measure dietary intake. However, as previously described in Chapter 1 and 2, the current methods for measuring flavonoid intake are flawed and there is an absence of dietary assessment tools for older Australians. Therefore, this chapter describes two studies to address the measurement of dietary flavonoids in older Australians. The first is a secondary data analysis of the Blue Mountains Eye Study, conducted to describe the intake of flavonoids and flavonoid subclasses, and the main dietary sources of these, in older Australian adults. Secondly, this chapter outlines the utilisation of this data in the development and subsequent validation of a food frequency questionnaire designed to easily estimate flavonoid intake in older Australians.

This chapter has been written and presented as two separate publications:
Appendix C

Kent, K, Charlton KE, Russell J, Mitchell P, Flood V, 2015. Estimation of flavonoid intake in older Australians: secondary data analysis of the Blue Mountains Eye Study. Journal of Nutrition in Gerontology and Geriatrics. http://dx.doi.org/10.1080/21551197.2015.1088917

Kent, K, Charlton, KE, 2015. Validation of a food-frequency questionnaire to measure flavonoid intake in older adults. (In preparation)

This data was also presented in:

Caldwell* K., Charlton K., Flood V., and Russell, J., 2014. Development of a Quantitative Food Frequency Questionnaire to Measure Flavonoid Intake in Older Australians. 24 and 25 of November 2013, Adelaide, Australia. Making

Research Matter Conference Proceedings: The 13th National Conference of Emerging Researchers in Ageing (pp. 44-44).

The Food Frequency Questionnaire developed and assessed in this chapter can be found in Appendix G.

PART 1: ESTIMATION OF FLAVONOID INTAKE IN OLDER AUSTRALIANS - SECONDARY DATA ANALYSIS OF THE BLUE MOUNTAINS EYE STUDY

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K.K. contributed to the design of the study, performed the analysis and prepared the manuscript; K.E.C. and J.R. provided advice on the study design and data analysis, and contributed to the preparation of the manuscript. P.W. and V.M.F. provided revision of the final manuscript.

3

3.1 Introduction

Information on consumption patterns of flavonoids is sparse, and only a few Australian estimations of flavonoid intake are available in the literature (1-3). Total flavonoid consumption (2), and the main sources of flavonoids (3), has been estimated in an Australian population using 24hr recall data from the 1995 National Nutrition Survey, in combination with the U.S. Department of Agriculture (USDA) flavonoid composition database (4). This analysis reported that the average adult intake of flavonoids was 454 mg day, with 70% of the intake attributed to black tea alone (3). The differing eating patterns of older Australians, compared with younger adults, were reflected in identification of different contributors of foods to total flavonoid intake. Notably, flavonoid contribution from tea and wine sources increased markedly with age (2). However, there are major limitations to the use of a 24hr recall for capturing the usual intake of flavonoids in a population. Some foods that are high in flavonoid content, such as seasonal fruits, may not be reflected and the high day-to-day variability in flavonoid intakes will introduce bias in estimation of habitual intakes. To this end, the National Nutrition Survey analysis provides an initial insight into the main sources of flavonoids and flavonoid subclasses in the Australian population, which differed from varied international estimates (5-9). In older adults few estimates of total flavonoid intake exist and a varied range of intake has been reported (8). The Australian population-based estimation has a limited focus on older adults and thus provides insufficient evidence of estimated flavonoid intake in older Australians. Therefore, despite a large body of evidence that links flavonoid consumption with improved health outcomes in older adults, there is little published information on quantities, subtypes and food sources of flavonoids in this age group.

The aim of this research was to describe dietary flavonoid intake in a sample of older Australians and identify the intake of flavonoids from various sub-classes (flavonols, flavan-3-ols, anthocyanins, flavones, and flavanones), using weighed food record (WFR) data obtained from a sub sample of older adults participating in the Blue Mountains Eye Study (BMES).

3.2 Methods

The BMES is a longitudinal, population-based cohort study of eye diseases and other chronic health outcomes in residents aged 49 years and over in a defined area, west of Sydney, Australia (16). The BMES is unique in that it collected detailed dietary information specifically in older Australian adults. To the best of our knowledge, this study provides the most comprehensive dietary information from older Australians to date. The original cohort (n=3654) was enrolled at baseline (1992-1994) and were followed up at 5 year intervals (1997-1999; 2002-2004; 2007-2009) (16). The dietary intake data utilized in this study was collected in 1994, and comprised three separate 4-day WFRs, that is twelve days in total, collected four months apart in a randomly selected sub-sample of the BMES cohort (n = 79), and thus reflected intake over a 12 month period, in different seasons (17-19). This dietary data was collected to assess the validity and reproducibility of a self-administered food frequency questionnaire developed to measure dietary patterns in the larger cohort (17). Of the 150 individuals that agreed to participate in the validation study, a total of 79 participants adequately completed three WFR over eight months(17). The sub-sample comprised 45 females (57%) and 34 males (43%) with a mean age of 70.1 years (age range was 60y to 80+y) (17) and a mean BMI of 21.27 (±3.3). WFR data was analysed using Microsoft Access (2010). The proportion of men and women who participated in the WFR study and the baseline BMES cohort were similar. By design, those who participated in the WFR validation study were older than those in the BMES cohort study (70.1 years to 65.4 years, $p<0.0001$). An older sample was selected to conduct the validation study as research interests were in age-related diseases uncommon in younger people and researchers wanted to ensure a valid instrument for subjects likely to become incident cases (17). The data was manually cross-referenced with the USDA Database for the Flavonoid Content of Selected Foods (Release 3.1) (10) to assign each food reported a total flavonoid value and a value for each flavonoid subclass: flavonols, flavan-3-ols, anthocyanins, flavones, flavanones. The isoflavone content of foods is not reported in the USDA flavonoid database (it is reported in a separate database). This point, coupled with the fact that isoflavone consumption in Australia is very low (14) (isoflavones are largely provided by soy foods), isoflavone intake was not assessed.

Data was exported from Microsoft Access to SPSS statistical program (V17.0: 2006, SPSS, Inc., Chicago, II, USA) for analysis. The mean and range of flavonoid intake per 4-day food record was calculated and intake of flavonoids and flavonoid subclasses per person, per day was estimated. Differences between males and females were analyzed using a one-way ANOVA. Differences between the 4day WFRs taken across three different time points were assessed using a one-way ANOVA. A p value ≤ 0.05 was considered to indicate statistical significance. The coefficient of variation was calculated for total flavonoid intake to analyze the degree of inter-individual variation between WFRs, and was reported as a percentage. The intra-individual variation was calculated to assess the potential variability within each individual's score at each WFR. The proportion that each flavonoid subclass contributed to overall flavonoid intake was determined by calculating the total reported amount (mg) of each subclass divided by the total amount (mg) of flavonoids reported (described as percentage). Rich and commonly consumed dietary flavonoid sources were identified and ranked in relation to their contribution to overall flavonoid intake in the cohort, by calculating the total reported amount (mg) of a flavonoid subclass present in each food (as per the USDA database) divided by the total amount (mg) of flavonoids reported in each subclass (described as percentage).

3.3 Results

Mean, standard deviation and range of intake for total flavonoids and for each flavonoid subclass, per person each day was calculated (Table 3-1).

There were significant differences between males (9130.3±3197.8 kilojoules/day) and females (7427.5±4699.1 kilojoules/day) for total energy intake (p=0.0005). However, there were no significant differences between males and females for total flavonoids (p=0.068) or for flavonoid subclasses; flavonol (p=0.621), flavone (p=0.364), flavanone (p=0.431), flavan-3-ol (p=0.055) and anthocyanin (p=0.081). The total percentage contribution of each flavonoid subclass to total flavonoid intake according to BMES WFR data is shown in Figure 3-1.

Table 3-1 Mean Intake of Total Flavonoids and Flavonoid Subclasses per Person, per Day According to Weighed Food Records*

	n	Flavonoid (total) mg /day	Flavonol mg /day	Flavone mg /day	Flavanone mg /day	Flavan3ol mg /day	Anthocyanin mg /day
Total	79	683.2±506.7 (7.3–5060.9)	28.7±20.4 (0.4–204.4)	1.9±2.9 (0–26.8)	21.2±25.4 (0–147.8)	629.4±494.1 (0-4906.3)	7.0±9.2 (0-69.4)
Male	34	616.9±435.4 (7.3-2038.2)	27.9±22.1 (0.375-204.3)	1.7±2.4 (0-16.1)	22.6±26.9 (0-147.8)	561.3±419.3 (0-1921.3)	8.1±1.0 (0-49.1)
Female	45	732.6±550.1 (38.7-5060.9)	29.2±19.1 (2.6-181.6)	2.1±3.1 (0-26.2)	20.1±24.3 (0-131.9)	680.2±538.9 (7.56-4906.3)	6.1±8.4 (0-69.4)

*Data are expressed as mean±SD (range: min - max).

One way ANOVA assessed the difference in total flavonoid intake and intake of flavonoid subclasses between the three separate 4-day WFRs and showed no significant differences in total intake or intake of any subclass between each of the collection periods. The inter-individual variation between WFRs showed a similarly large variation for WFR1 (55%), WFR2 (53%) and WFR3 (57%). The coefficient of variation, which assessed the intra-individual variation in flavonoid intake across the three separate 4-day WFRS, showed a mean variation of 28.6% (range 0.9%-122.9%, median 22.2%).

The total number of reported foods in the collated BMES WFR data was 895 foods and flavonoid content was assigned to 171 of these foods. The sources of total flavonoids that contributed the most to each food group as reported in the BMES WFR data, were calculated (Table 3-2). The top five foods that contributed to the flavan-3-ols, flavonols, flavones, flavanones and anthocyanin subclasses of flavonoids are shown in Table 3-3.

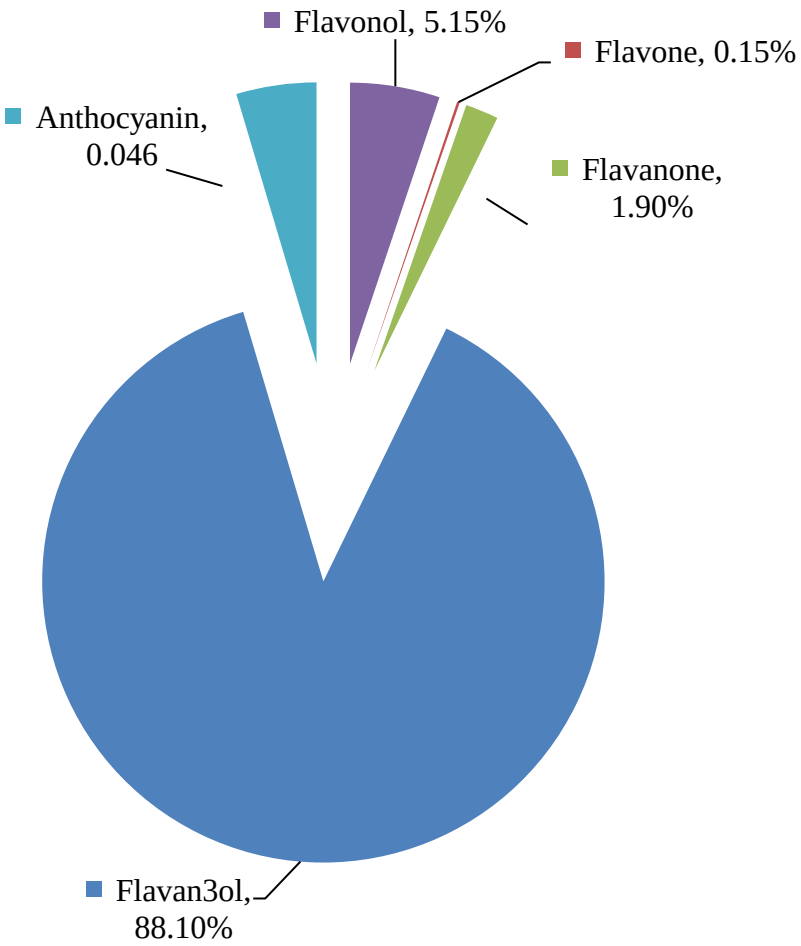

Figure 3-1 Percentage contribution of flavonoid subclasses to total flavonoid intake.

Table 3-2 Top 10 Flavonoid Containing Foods Ranked in Order of Contribution to Total Consumption

	Fruit		**Vegetables**		**Beverages**		**Alcohol***		**Other foods**	
	Food (mean serve, g)	%	Food (mean serve, g)	%	Food (mean serve, g)	%	Food (mean serve, g)	%	Food (mean serve, g)	%
1	Orange (129.0)	1.37	Onion (63.5)	0.34	Black tea (237.6)	89.1	Wine, Red (128.0)	0.44	Parsley (13.94)	0.18
2	Banana (100.7)	0.86	Tomato, raw (59.5)	0.19	Green tea (307.4)	1.3	Wine, White (140.9)	0.06	Marmalade (19.4)	0.08
3	Apple , Raw (112.0)	0.66	Green Bean (187.84)	0.12	Orange Juice (127.9)	1.1	Beer (151.2)	0.04	Milo Powder (12.4)	0.03
4	Apple, Stewed (110.7)	0.12	Potato (104.8)	0.09	Grapefruit Juice (137.1)	0.09	Sherry (73.1)	0.02	Chocolate, Dark (18.3)	0.019
5	Mandarin (93.5)	0.12	Spinach (66.5)	0.05	Apple Juice (148.6)	0.06	Champagne (271.1)	0.002	Almond (24.8)	0.011
6	Peach (125.3)	0.12	Brussels Sprout (67.6)	0.04	Coffee (188.9)	0.06			Pecan (34.0)	0.08
7	Strawberry (99.4)	0.09	Pumpkin (66.21)	0.04	Lemon Juice (24.6)	0.04			Date (36.4)	0.007
8	Grape, black (160.9)	0.08	Radish (47.7)	0.04	Milk, chocolate (256.5)	0.02			Cocoa powder (4.31)	0.065
9	Grapefruit (79.3)	0.06	Celery (53.5)	0.04	Tomato Juice (235.6)	0.006			Jam, Berry (24.75)	0.004
10	Plum (121.1)	0.06	Lettuce (42.1)	0.03	Grape Juice (102.55)	0.001			Ginger (10.1)	0.004

% = percentage contribution to overall flavonoid intake according to 12 days of WFRs (n=79) from participants in the Blue Mountains Eye Study; * Alcohol only had five contributing beverages.

Table 3-3 The Five Main Dietary Sources of Each Flavonoid Subclass Based on Weighed Food Records

Flavonoid Subclass	Food	% contribution to subclass
Flavan-3-ols	Tea, Black	94.55
	Tea, Green	3.52
	Apple	0.40
	Banana	0.39
	Wine, red	0.155
Flavonols	Tea, Black	68.59
	Onion	7.73
	Apple	3.39
	Tea, Green	2.78
	Beans	2.76
Flavones	Parsley	98.52
	Tomato	0.69
	Celery	0.27
	Pumpkin	0.23
	Watercress	0.13
Flavanones	Oranges	44.47
	Orange Juice	36.78
	Mandarin	4.07
	Tomato	3.06
	Grapefruit Juice	2.80
Anthocyanins	Blueberry	29.56
	Banana	22.81
	Radish	9.14
	Wine, red	8.19
	Cabbage, red	7.71

3.4 Discussion

The BMES WFR data was derived from 12 days of weighed food records in 79 people, equating to 948 person-days analysed. The mean intake of total flavonoids (683mg/day) was significantly higher than previous estimations (454mg/day) (2) for the total Australian population. However, it is similar to the estimation of flavonoid intake (693mg/day) for the 65+ year old age group within the study from the Australian population estimates (2). The proportional intake of each flavonoid subclass in our data (Figure 3-1) compares closely with the Australian nationally representative 1995 National Nutrition Survey (NNS) data that was based on a 24hr recall method (3). Flavan-3-ols contributed the majority of flavonoid intake (92%), followed by flavonols (4%), flavanones (3%) and flavones (<1%). The difference in total flavonoid intake estimated between the studies may reflect the difference in dietary assessment methods and the size of the study population, however, the agreement seen between the comparative age group may confirm a higher flavonoid intake in this age range.

The major food sources of flavonoids identified by the current analysis were black tea (89.1%), oranges and orange juice (1.37% and 1.3% respectively), green tea (1.3%) and bananas (0.87%). The strength of this data relates to the 12 days of dietary data being collected over 12 months, to reduce the impact of seasonality of food consumption and consequently flavonoid intake. However, it is interesting to note that no difference was seen in total flavonoid intake or intake of any flavonoid subclass between the three separate 4-day WFR, when comparing the group intakes as a whole. This is likely to be attributed to the habitual nature of tea consumption, which is fairly stable throughout the year. However, the high intra-individual variation, where on average there is a variation of nearly 29% in flavonoid intake between the 3 separate 4-day WFRs, is quite significant. Therefore, the impact of seasonality for the group as a whole may be low, but the extent to which a particular individual may fluctuate is quite substantial. This should be a consideration in future studies investigating dietary flavonoid intake, whereby collecting dietary data in different seasons may not significantly influence flavonoid intake estimates on the population level, but the importance of individual variation should be considered.

The food sources identified are similar to the foods reported in the Australian NNS95 population based analysis (3). In another Australian study (1), similar food sources were reported to contribute to flavonoid subclasses in a group of healthy, young women. Major sources of flavonols were reported to be tea, apples and onions, while major sources of flavones were parsley and celery, and significant sources of flavanones were citrus fruits, including oranges and grapefruit. Additionally, tea was shown to be the main contributor to total flavonoid intake (1), a finding confirmed by our study.

International comparisons identify large variations in estimates of flavonoid intakes of older adults, with one US study reporting adults aged ≥51 years to consume only 191mg/day, as estimated from two 24h diet recalls (15). A Finnish study of males aged 48, 54 or 60 reported a similarly low estimate of 139mg/day, based on a 4-day food record (16). However, the trend showing higher intake of dietary flavonoids in older adults compared with other age groups is similar across all the national and international estimates. The low flavonoid intake estimates may be attributable to different dietary assessment methods and may also be potentially linked to the application of more limited flavonoid reference databases, leading to an under-reporting of total flavonoid intake.

While there was no significant difference between genders for total flavonoid intake or intake of any flavonoid subclasses, a difference in total energy intake was noted. This indicates that the major contributors to flavonoid intake may be minor contributors to total energy intake. This is highlighted by the influence of black and green tea, which account for more than 90% of total flavonoid intake and are low in kilojoules. Future research could adjust for energy intake to assess whether there are differences in flavonoid intake between genders after accounting for differences in energy intake.

Despite providing sound dietary data, there are several limitations identified with utilizing the BMES WFR data to estimate flavonoid intake in older Australians. Firstly, this data was collected in the mid-1990s and the diets of older adults may have

evolved or changed over time to present. There is, however, a notable lack of population-based studies in older Australian adults that has collected dietary information with this level of precision since that time. A 10-year follow up of the BMES cohort showed overall fruit and vegetable consumption had not significantly changed from baseline (17). Changes that were found in dietary patterns over time generally related to fat (MUFA, PUFA, and SFA) and total sugar (not CHO) intake. These macronutrients are not present in large quantities in flavonoid-rich foods. Despite little change in fruit and vegetable intake over time in cohort survivors, applicability of the study findings may be limited by the changing food supply, limiting the generalizability of this data to the current population for specific food groups. However, further research is required to confirm these assumptions. While the BMES study population was generally representative of the older Australian population, they had slightly a higher socioeconomic status and a slightly higher level of post-school qualifications than the general population (18). Additionally, people who agree to complete 12 days of WFRs are also more likely to be highly motivated about recording the details of their diet (18), and could also therefore tend to have higher quality diets with an increased nutrient density, compared to the general population. Lastly, this secondary data analysis relied on the WFR data which had previously been transcribed into Diet 1.3 software (19). This software was not designed to incorporate phytochemical information and consequently there are limitations associated with our ability to manually assign flavonoid values to foods. One major example of this is green tea, which is combined with other herbal teas in the food composition databases. Despite perhaps having similar nutritional properties in terms of nutrients, the phytochemical properties of different herbal teas are extremely varied (20).

Another major limitation relates to utilisation of the USDA database to determine the flavonoid content of selected foods as a reference database. The flavonoid content of foods in the database may not reflect that of Australian produce, as flavonoid content is heavily influenced by growth and processing conditions and the USDA recognizes this variability (4). Another well-established limitation relates to the inability of a reference database to account for storage and cooking methods (4). Future research should attempt to account for this through the application of retention factors for some

food preparation techniques, including boiling. However, retention factor applications in this instance were not warranted, as full disclosure of food treatment and cooking was not available due to limitations associated with secondary data analysis. Additionally, bioavailability of flavonoids is not taken into account, despite showing extreme individual variability (21). Once retention factors for all foods are perfected and the metabolism of flavonoids is adequately understood, they should be primary considerations in estimating bioavailability and therefore the bioactivity of flavonoids. It is also important to highlight that the USDA database attributed a delphinidin value for bananas of 7.39 mg/100 g, which may incorrectly reflect banana proanthocyanidin degradation during content analysis (22). However, all values that were presented in the USDA database were accepted as it was outside the scope of this analysis to question a critically evaluated and widely used database; however, this limitation means anthocyanin intake may be overestimated in this cohort.

A further limitation of this study relates to the measurement of flavonoids alone, as many other classes of polyphenolic compounds hold similar biological properties. Total flavonoid intake and the intake of total flavonoid subclasses only were measured instead of specific flavonoids, in order for the results to have a more general application in broad nutrition research. Additionally, it is likely that specific flavonoids within a subclass have similar bioactivities.

3.4.1 Conclusion

Accurate estimation of flavonoid intake in populations is an important methodological consideration for studies that investigate the health impact of these food-derived bioactive compounds. Our analysis provides an assessment of usual intakes of flavonoids that may be useful for planning intervention studies in which dose-response outcomes are of interest.

3.5 References

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PART 2: DEVELOPMENT AND RELATIVE VALIDATION OF A FOOD-FREQUENCY QUESTIONNAIRE TO MEASURE FLAVONOID INTAKE IN OLDER ADULTS

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KK was responsible for the development of the study, organisation and leading of the data collection and analysis, and preparation of the manuscript. KEC was responsible for the design of the study and analysis, and critical revisions on the manuscript.

The Food Frequency Questionnaire can be found in Appendix G.

3

3.1 Introduction

Currently, the consumption patterns of flavonoids in older adults have not been adequately assessed, and only a few Australian estimations are available in the literature (1, 2). The dietary assessment instruments currently used to measure flavonoid intake have limitations in their ability to accurately assess habitual intake, which hinders investigation of the association of the health benefits of flavonoids in epidemiologic studies (3). Inconsistent methodologies have been used to quantify flavonoid intake and few survey tools have been validated for this purpose (4). The collection of dietary information using food recall or food record (FR) methods has been employed most frequently when determining intake of flavonoids, and is cross-referenced with flavonoid-specific food composition databases. However, the burden of these methods can be large on both participants and researchers.

Previously published food frequency questionnaires (FFQ) that have been developed to measure flavonoids have focused on specific flavonoids or flavonoid subclasses, rather than a comprehensive range to reflect total dietary intake. Hakim et al. (5) validated a FFQ that assessed intake of tea and tea polyphenols against a standard FFQ and 4-day FRs. In a more focused study, Hakim et al. (6) measured D-limonene contents in a range of citrus juices as the basis for the assessment of the intake of this flavonoid. Although accurate, if these types of instruments were expanded to provide information on the full range of flavonoids, their time burden on the respondent and researcher would be excessive. Research has also utilised post-hoc analysis of pre-existing FFQs that were not developed or validated for the purpose of measuring flavonoid intake (7, 8). This method has demonstrated large limitations in accurately assessing flavonoid consumption, because of grouping foods that may have similar nutritional profiles (pears and apples) and appear sensible to the respondent to consider together when reporting their intake, but which have vastly different flavonoid contents.

Recent Australian research has validated a flavonoid specific FFQ in young adults, against 3 day FRs (9). Additionally, total flavonoid consumption (1), and the main sources of flavonoids (4), have been estimated in an Australian population using 24hr recall data

from the National Nutrition Survey, 1995. The different eating patterns of older Australians was reflected in different dietary sources of flavonoids compared to younger adults, whereby contributions from tea and wine flavonoids increased markedly with age (1). Given the differences in total flavonoid intake, as well as the different major dietary sources of flavonoids in the diets of young and older adults (1), it is important to develop and validate a FFQ to measure the intake in older adults specifically. Despite a large body of evidence that links flavonoid consumption with improved health outcomes in older adults (10), there is little published information on habitual intake of food sources of flavonoids in this age group. A flavonoid specific FFQ, that includes a list of both flavonoid rich and the most commonly consumed food items that contribute to total intake, would be a useful contribution to research that investigates flavonoid consumption in older Australian cohorts, for the purpose of exposing diet-health associations, as well as to inform the development of dietary guidelines. The advantages of a FFQ over dietary recall and diary methods to assess long term intake patterns of nutrients, and to a lesser extent, foods and food groups, is well established (11). A FFQ is a cheaper and markedly less burdensome method for measuring dietary exposure to dietary attributes (12).

A focused retrospective analysis of dietary flavonoid intake older adults was necessary to inform the development of a novel dietary assessment tool (2). We recently estimated the mean intake of total flavonoids, and the major dietary sources thereof, in an older Australian population using twelve days of weighed FRs (2), collected as a part of the Blue Mountains Eye Study (13). These findings (14) were utilised to develop a FFQ for use in older adults, the validation and reproducibility of which is the objective of this study. Validity, in this context, is the degree to which the FFQ measures specific dietary attributes, and the validation process involves appraisal of the FFQ against a reference method to determine their agreement in measuring the same dietary attributes (15). The reproducibility of an FFQ relates to the ability of the tool to accurately and equally measure the same dietary attributes on more than one occasion (15).

The aim of this study was to assess the validity and reproducibility of a FFQ developed to measure total flavonoid intake, and flavonoid subclasses, in older adults against a 4-day FR.

3.2 Methods

3.2.1 Development of the FFQ

A FFQ was developed to quantitatively assess individual intake of dietary flavonoids over 12 months and is structurally based on the National Health and Nutrition Examination Survey (NHANES) FFQ (16). All points of recommendation as outlined by Cade et al., (11) were addressed in the development of the instrument.

As reported elsewhere (2), 12 days of weighed FR data from the Blue Mountains Eye Study (BMES) were used as the main basis for the development of the FFQ. To ensure all rich sources of flavonoids and top contributors to flavonoid intake were captured; foods were selected for inclusion in the FFQ if they met the following criteria:

1. Any food with >30mg/100g total flavonoids and/or >30mg/average portion size.
2. Any flavonoid containing food in the top 25% of all foods.
3. A top 25% contributor in each flavonoid subclasses according to the average portion size (g) of that food.

Several additional inclusions from the USDA database of flavonoid containing foods were selected on the basis of the definition of a flavonoid rich food, >30mg/100g edible weight, and if they were available in Australia and could form part of usual cuisine. Additionally, any foods highlighted as top contributors of flavonoid intake by previous Australian literature (1, 4) were also included. Of the total 96 foods included in the FFQ, 73 foods were identified from the BMES WFR data. An additional 23 foods were included from rich sources identified from the USDA flavonoid database (Table 3-1) and from other current literature sources (Table 3-2).

The USDA database for the flavonoid content of selected foods was selected as the reference database for this study, as it is one of the most complete sources of flavonoid content data. The lack of an equivalent Australian database for the assessment of flavonoid intake from dietary records necessitated the use of an international one. While other databases, including PhenolExplorer and EURO-FIR-eBasis. As the USDA database was adequately comprehensive (i.e. all included foods were able to be assigned flavonoid content), no other flavonoid databases were consulted.

Table 3-1 Rich flavonoid sources (>30mg/100g) identified from the USDA database (3.1) (12).

Food source	Flavonoid content/100g
Dried Parsley	215.46
Dill	55.15
Thyme	45.25
Capers (canned)	259.19
Mint	30.92
Coriander	52.9
Lime	43.0
Eggplant	85.69
Blackcurrant juice	45.27
Red onion (raw)	39.21
Rocket	66.19
Oregano	311.73

Table 3-2 Flavonoid sources identified from Australian literature sources (2, 5).

Food source	Flavonoid content/100g
Cherries	30.21
Lemon	53.38
Silverbeet (chard)	1.75
Endive	10.1
Beans (broad)	0.9
Spring onion	30.6
Mango	2.0
Rhubarb	2.35
Olives (green)	0.56
Olives (black)	4.93
Kale	92.98

Grouping of food items was decided *a priori* to the analysis, with items being coded into the food categories of beverages, alcohol, fruit, vegetables, herbs and other foods. With

the aim of accurately quantifying flavonoid intake, serving sizes derived from the BMES WFR data were used (2). Median portion size (g) and the inter-quartile range informed the basis for four portion size options, specific to each food. A time period of 12 months was selected to ensure that habitual intake and the influence of seasonal dietary change was captured in the FFQ.

3.2.2 Subjects

A total of 42 community dwelling older adults aged 60 years and over from the Illawarra region of NSW, Australia, were recruited to the study between June and September 2014. Generally healthy older adults were recruited via advertising material distributed at local community groups. The study excluded individuals with major food intolerances or a condition that impacts usual diet (e.g. Cohn's disease). Ethics approval was obtained from the University of Wollongong Human Research Ethics Committee and all subjects provided written informed consent. To determine an adequate sample size, a previous study (17) which assessed the validity of an FFQ against multiple 24h recalls, minimum of approximately 42 subjects were required to obtain a correlation coefficient of 0.377.

Participants attended two identical interviews, held one month apart, and based on their preference for either a home visit or attendance at the university testing facility (Illawarra Health and Medical Research Institute). A single interviewer (KK) conducted the interview, at which weight (kg) and height (m) was measured and body mass index (BMI; weight (kg) / height (m) ^{2}) was calculated. Resting blood pressure (BP) and heart rate (HR) was measured while seated, in triplicate and averaged using an Omron HEM7200 Deluxe Automatic Monitor. Personal and demographic information was collected including age, smoking and drinking habits, and education level.

3.2.3 FFQ Analysis

The self-administered FFQ, completed by the participant at home on the day before the interview, was screened during the interview for errors and missing data. Any questions about the foods contributing to the FFQ were clarified. The paper-based FFQ data was entered into a template created for this purpose in Microsoft Excel (Microsoft version 14.1.2). The template was developed using nutritional information for each food or

beverage, for each serving size option in the FFQ, from the Foodworks dietary analysis package (Xyris software, version 5, 2007, Highgate Hill, QLD, Australia) and USDA database for the flavonoid content of selected foods (18). Foods reported were converted to daily intake frequencies as follows: 6+ per day= 7.5; 4-5 per day = 4.5; 2-3 per day = 2.5; 1 per day = 1; 5 - 6 per week; 0.8; 3-4 per week = 0.5; 1-2 per week = 0.2; 2 - 3 per month = 0.09; 1 per month or less = 0.04; Never = 0.

3.2.4 Dietary Assessment

Participants were briefly trained by a nutritionist (KK) and instructed to complete a 4-day FR on any four consecutive days between the two interview dates, including 3 week days and 1 weekend day. Participants were instructed to record in detail all the foods and drinks they consumed during that period and to estimate the portion sizes using cup measures, weights or other household measurements. Dietary data from the 4-day FR was screened during the final interview and analysed using the Foodworks dietary analysis package (Xyris software, version 5, 2007, Highgate Hill, QLD, Australia). As dietary data relating to flavonoid content of foods is not integrated into the Xyris software, the food items were manually cross-referenced with the USDA database for the flavonoid content of selected foods (18) to estimate flavonoid consumption.

3.2.5 Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) V15.0.0 (SPSS Inc., Chicago IL, USA). Descriptive statistics were performed on demographic data. For each participant, mean (±SD) flavonoid intake, intake of each flavonoid subclass from FFQ1, FFQ2 and 4-day FR was calculated. From this the mean (±SD) intake per person/per day for each method was determined. Major dietary sources of flavonoids and each flavonoid subclass were determined. Mean daily flavonoid intakes for the FFQ1, FFQ2 and 4-day FR were assessed for normality using histograms and the Shapiro Wilk test for normality. Data was not normally distributed for FFQ1, FFQ2 and 4-day FR.

3.2.6 Validity

A combination of tests was applied to assess several different facets of validity, which may therefore provide a superior assessment of validity than a single measure (19). A Wilcoxon signed rank sum test was used to compare flavonoid and flavonoid subclass intakes obtained from the two methods (FFQ1 and 4-day FR). Spearman correlation coefficients were calculated between the two dietary assessment methods (i.e. FFQ1 and FR) for total flavonoid intake and each dietary flavonoid subclass to test the strength of a relationship between two variables.

The FFQ1 and 4-day FR were assessed for level of agreement using Bland-Altman plots (20), where the difference between the FFQ1 and 4-day FR (FFQ1 - 4-day FR) was plotted against the mean of the FFQ1 and 4-day FR (FFQ1 + 4-day FR)/2. Limits of agreement (the mean difference±1.96SD) for the difference between the two measures were calculated to evaluate if they were acceptable. A regression line was fitted in the graph to detect proportional differences and to indicate the direction and magnitude of the bias.

Lastly, the Cohen's kappa (κ) test determined the FFQ's ability to rank individuals into quartiles of intake (FFQ1 vs 4-day FR) for total flavonoid intake and flavonoid subclasses (21, 22), whereby values ≤ 0 as indicating no agreement and 0.01–0.20 as none to slight, 0.21–0.40 as fair, 0.41–0.60 as moderate, 0.61–0.80 as substantial, and 0.81–1.00 as almost perfect agreement.

3.2.7 Reproducibility

A combination of tests was applied to assess reliability (19). The mean percentage difference between FFQ1 and FFQ2 for total flavonoid intake and intake of flavonoid subclasses was calculated. Wilcoxon signed rank sum test was used to compare flavonoid and flavonoid subclass intakes obtained from the FFQ1 and FFQ2. The association between FFQ1 and FFQ2 was assessed using the Spearman correlation for total flavonoid intake and each flavonoid subclass. Bland-Altman graphs were drawn for the difference between FFQs (FFQ1 - FFQ2) plotted against the mean of the FFQs ((FFQ1 + FFQ2)/2). Limits of agreement (the mean difference±1.96SD) for the difference between the two measures were calculated to evaluate if they were acceptable and to indicate the direction and magnitude of the bias. A regression line was fitted in the graph to detect proportional differences. The Cohen's kappa (κ) was determined to assess the FFQ's reliability to

ranking individuals into quartiles of intake (FFQ1 vs FFQ2) (21, 22), whereby values ≤ 0 as indicating no agreement and 0.01–0.20 as none to slight, 0.21–0.40 as fair, 0.41–0.60 as moderate, 0.61–0.80 as substantial, and 0.81–1.00 as almost perfect agreement.

3.3 Results

The developed flavonoid FFQ (Appendix G) includes 93 questions, pertaining to 9 non-alcoholic beverages, 5 alcoholic beverages, 25 fruit, 33 vegetables, 8 herbs, 13 other foods (dried fruit, nuts, fruit spreads), and an additional 3 summary questions about usual alcohol intake, and fruit and vegetable consumption. The characteristics of the study participants are presented in Table 3-3.

Table 3-3 Summary statistics of subject characteristics (n=42).

Gender	Male	14 (33.3%)
	Female	28 (66.7%)
Age (y)	Mean±SD	75.3 ± 8.6
BMI (kg/m^2)	Mean±SD	27.7 ± 4.3
Primary Language	English	40 (95.2%)
	Other	2 (5.8%)
Smoking status	Non-smoker	28 (66.7%)
	Current smoker	1 (2.4%)
	Ex-smoker	13 (30.9%)
Supplement Use	Any supplement	24 (57.2 %)
	No supplement	14 (42.8%)
Education (highest level completed)	Tertiary	27 (64.3%)
	Secondary (complete)	15 (35.7%)
	Secondary (incomplete)	0
	Primary	0
Blood Pressure	Systolic Mean±SD	138.1 ± 18.4
	Diastolic Mean±SD	83.74 ± 9.3
	Heart rate: Mean±SD	67.2 ± 10.6

The mean intake, standard deviation and range of total flavonoid intake and intake of each flavonoid subclass (mg/day) for each method were determined (Table 3-4). For both the

FFQ (1 and 2) and the 4-day FR, a large variability in dietary flavonoid intake and intake of flavonoid subclasses is described (Table 3-4). The percentage contribution that each food made to total flavonoid intake and intake of each flavonoid subclass was calculated for each person (FFQ1) and averaged to indicate the major (top 10) foods contributing to intake in the total group (Table 3-5).

Table 3-4 Description of mean flavonoid intake (mg/day) according to the FFQ1, FFQ1 and 4-day FR (n=42)

	(mg/day)	**Mean**	**Std. Deviation**	**Minimum**	**Maximum**	**% contribution**
FFQ 1	Total Flavonoids	1050.4717	725.52905	80.02	3253.48	
	Anthocyanins	103.3685	104.66311	11.45	368.27	9.84
	Flavan-3-ols	829.5545	690.60154	32.27	2953.08	78.96
	Flavanones	42.4992	28.47302	2.39	105.35	4.04
	Flavones	6.5072	8.03556	.27	37.74	0.61
	Flavonols	68.5423	34.99465	19.68	147.20	6.52
FFQ 2	Total Flavonoids	1047.0211	724.85865	140.14	2488.98	
	Anthocyanins	103.4143	99.03593	14.75	367.82	9.88
	Flavan-3-ols	829.7809	686.56737	60.92	2312.23	79.25
	Flavanones	40.4190	29.37282	4.87	103.56	3.86
	Flavones	7.3939	9.20604	.46	37.70	0.71
	Flavonols	66.0130	32.80539	16.44	131.84	6.30
4-day FR	Total Flavonoids	847.5078	534.75680	22.08	2490.47	
	Anthocyanins	38.8653	58.98484	.00	224.65	4.59
	Flavan-3-ols	754.6830	502.21665	6.93	2366.47	89.05
	Flavanones	18.5490	20.92953	.00	69.78	2.19
	Flavones	3.7307	5.24328	.00	27.40	0.44
	Flavonols	32.7868	18.88830	1.16	99.38	3.87

Table 3-5 Top 10 foods contributing to total flavonoid and flavonoid subclass intake ranked in order of contribution to total consumption according to the developed FFQ.

	Flavonoid Total		**Anthocyanin**		**Flavan-3-ol**		**Flavanone**		**Flavone**		**Flavonol**	
	Food	%	Food	%	Food	%	Food	%	Food	%	Food	%
1	Tea, black	56.97	Wine, red	31.13	Tea, black	75.22	Orange juice	40.86	Parsley	35.50	Tea, black	29.03
2	Wine, red	7.63	Grapes, red	13.29	Tea, green	7.42	Mandarin	19.18	Wine, red	17.06	Onion, brown	9.79
3	Tea, green	6.05	Cherry	10.28	Broccoli	4.80	Oranges	17.59	Pumpkin	11.08	Beans	8.93
4	Orange	3.33	Blueberry	7.94	Apple, red	2.48	Wine, red	6.75	Grapes, red	7.35	Peas	5.04
5	Broccoli	2.57	Banana	7.04	Peach	1.46	Wine, white	3.64	Tea, green	4.22	Onion, red	4.44
6	Grapes, red	2.08	Pear	5.96	Wine, red	1.42	Brussels sprout	3.59	Celery	4.02	Wine, red	4.26
7	Mandarin	2.07	Strawberry	5.53	Banana	1.06	Grapefruit	2.46	Spinach	2.30	Tea, green	3.81
8	Cherry	1.95	Apple red	3.78	Nectarine	1.02	Lemon, flesh	2.26	Orange	2.28	Apple	3.37
9	Apple, red	1.86	Plum	2.72	Cherry	0.94	Tomato	1.63	Kiwi fruit	1.93	Kale	2.66
10	Banana	1.10	Peach	1.47	Grapes, green	0.65	Lemon juice	0.67	Rock melon	1.83	Spinach	2.60

3.3.1 Validity

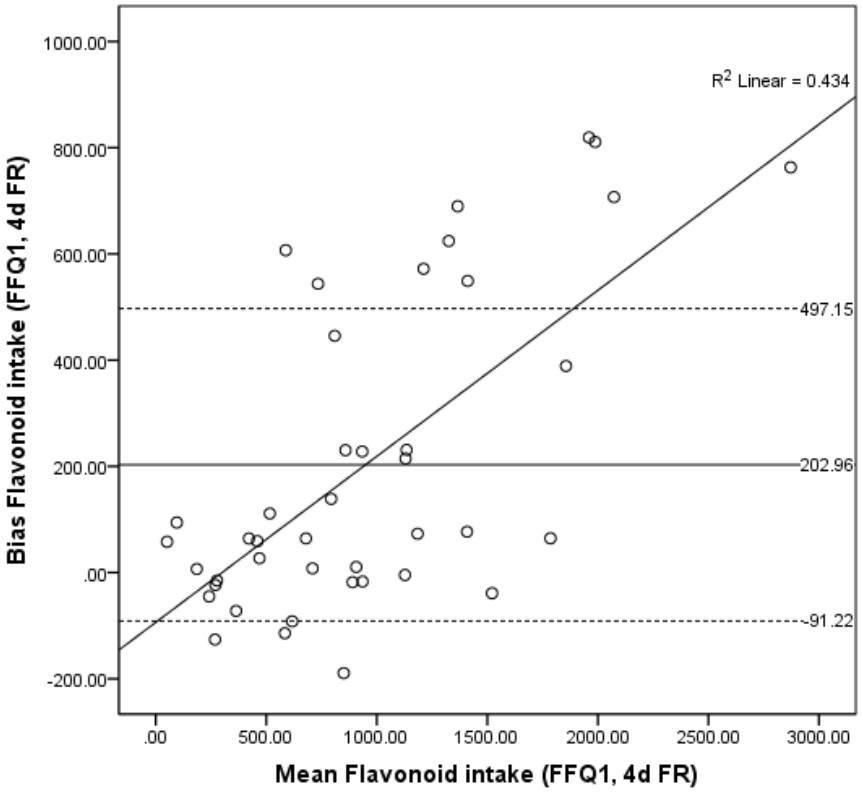
Wilcoxon signed rank sum test showed significant differences in total flavonoid ($p<0.001$), anthocyanin ($p<0.001$), flavanone ($p<0.001$) and flavonol ($p<0.001$) intakes as measured by the FFQ1 and 4-day FR, and no significant difference for flavone and flavan-3-ol measurements (Table 3-6). The strength of the relationship as tested by Spearman's correlation coefficients between the FFQ1 and 4-day FR are shown in Table 3-6.

The Bland-Altman plot of the bias (average of the differences between methods) against the mean value for the two methods is shown in Figure 3-1. Visually, this demonstrates that the bias and limits of agreement are large for total flavonoid intake (+202.96), and intake of anthocyanins (+64.5), flavan-3-ols (+74.85), flavanones (+ 23.9) and flavonols (+35.75). The bias is small, with narrow limits of agreement for flavone intake (+2.77). These results indicate that the FFQ1 and 4-day FR are systematically producing different results. Interpretation of the systematic bias shows that the FFQ is overestimating flavonoid intake (and intake of flavonoid subclasses) in comparison to the 4-day FR. The linear regression analysis indicates a strong positive correlation for total flavonoid intake, and intake of anthocyanins, flavan3ols and flavonols, whereby the greater an individual estimates their flavonoid intake, the greater the systematic bias (or overestimation by the FFQ). This relationship is lesser for flavone and flavanone intakes, as shown visually in Figure 3-1.

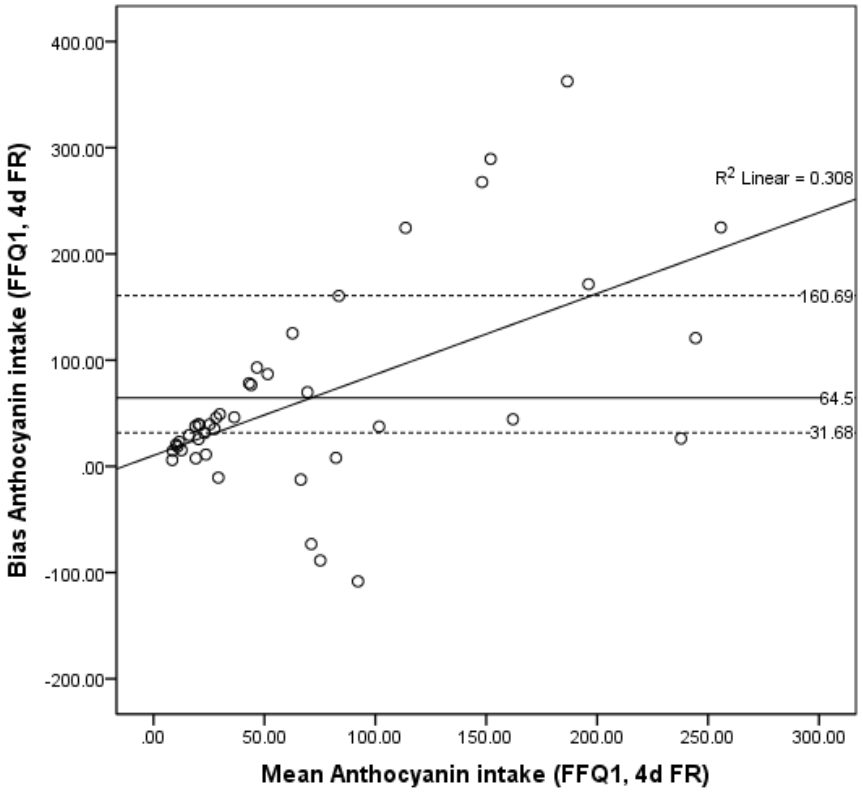
The Cohen's kappa (κ) indicates that there was substantial agreement (21) between the FFQ1 and 4-day FR for total flavonoid intake ($\kappa = 0.619$, $p<0.001$), moderate agreement for flavan-3-ol intake ($\kappa=0.492$, $p<0.001$) and flavonol intake ($\kappa = 0.619$, $p<0.001$); fair agreement for flavone intake ($\kappa=0.238$, $p=0.008$); slight agreement for anthocyanin intake ($\kappa=0.142$, $p=0.111$); and poor agreement for flavanone intake ($\kappa=0.048$, $p=0.587$).

Table 3-6 Comparison of the total flavonoid intake and intake of flavonoid subclasses (mg/day) for FFQ-1 vs FR (validity) and FFQ-1 vs FFQ-2 (reliability)

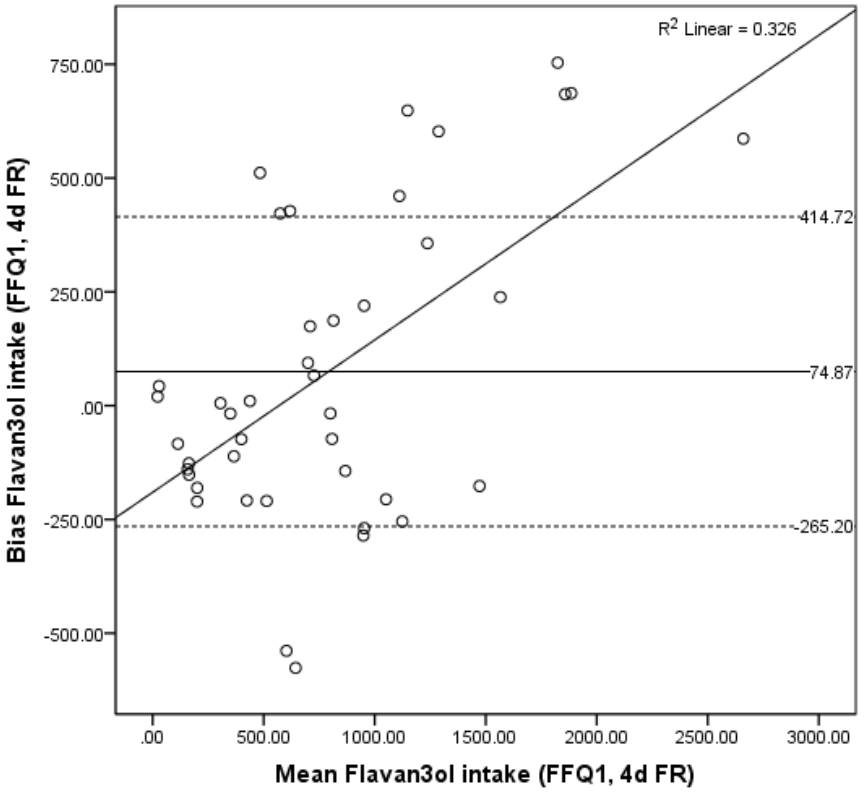
(mg/day)		**Wilcoxon signed rank sum test** Sig. (p-value)	**Spearman's Correlation Coefficient**	Sig. (p-value)
FFQ-1 - vs 4-day FR	Total flavonoids	<0.001	0.93	<0.001
	Anthocyanins	<0.001	0.32	0.042
	Flavan-3-ols	0.413	0.87	<0.001
	Flavanones	<0.001	-0.17	0.27
	Flavones	0.004	0.18	0.25
	Flavonols	<0.001	0.75	<0.001
FFQ-1 vs FFQ -2	Total flavonoids	0.912	0.91	<0.001
	Anthocyanins	0.955	0.92	<0.001
	Flavan-3-ols	0.957	0.92	<0.001
	Flavanones	0.350	0.73	<0.001
	Flavones	0.119	0.85	<0.001
	Flavonols	0.328	0.90	<0.001


Bias Flavonoid intake (FFQ1, 4d FR)
Mean Flavonoid intake (FFQ1, 4d FR)
R2 Linear = 0.434
497.15
202.96
-91.22
1000.00
800.00
600.00
400.00
200.00
.00
-200.00
.00
500.00
1000.00
1500.00
2000.00
2500.00
3000.00

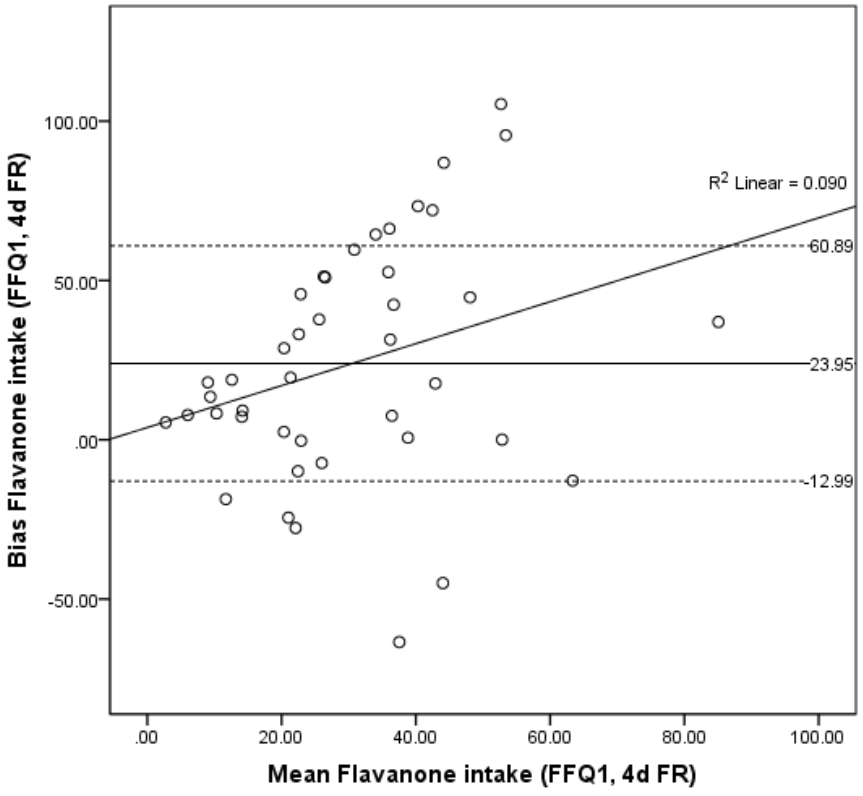
(a)


Bias Anthocyanin intake (FFQ1, 4d FR)
Mean Anthocyanin intake (FFQ1, 4d FR)
R2 Linear = 0.308
160.69
64.5
31.68
400.00
300.00
200.00
100.00
.00
-100.00
-200.00
.00
50.00
100.00
150.00
200.00
250.00
300.00

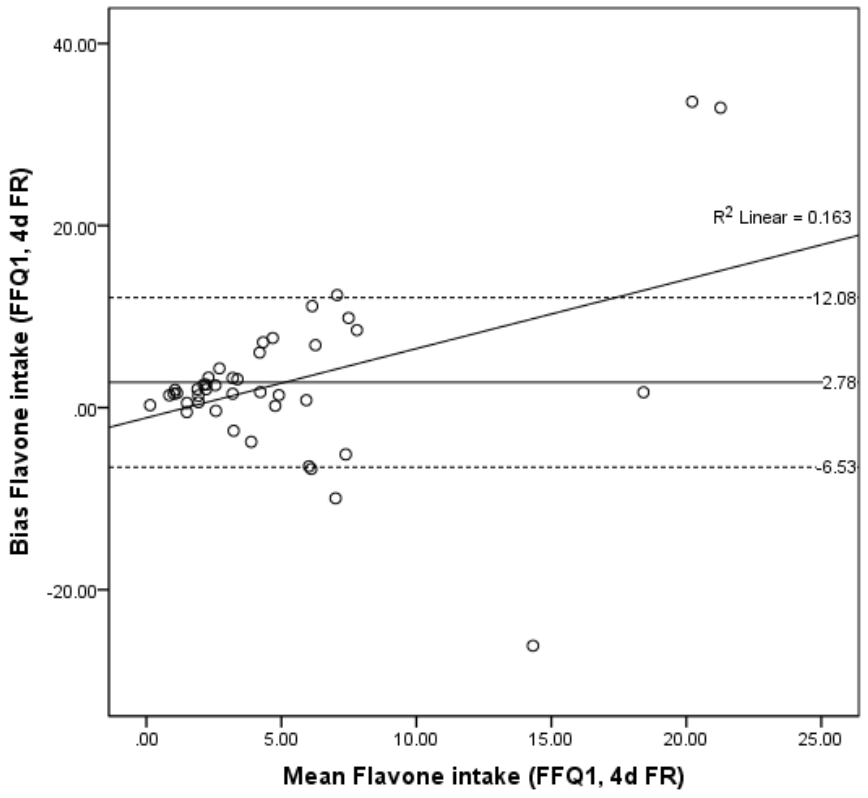
(b)


R2 Linear = 0.326
750.00
500.00
250.00
.00
-250.00
-500.00
414.72
74.87
-265.20
.00
500.00
1000.00
1500.00
2000.00
2500.00
3000.00
Bias Flavan3ol intake (FFQ1, 4d FR)
Mean Flavan3ol intake (FFQ1, 4d FR)

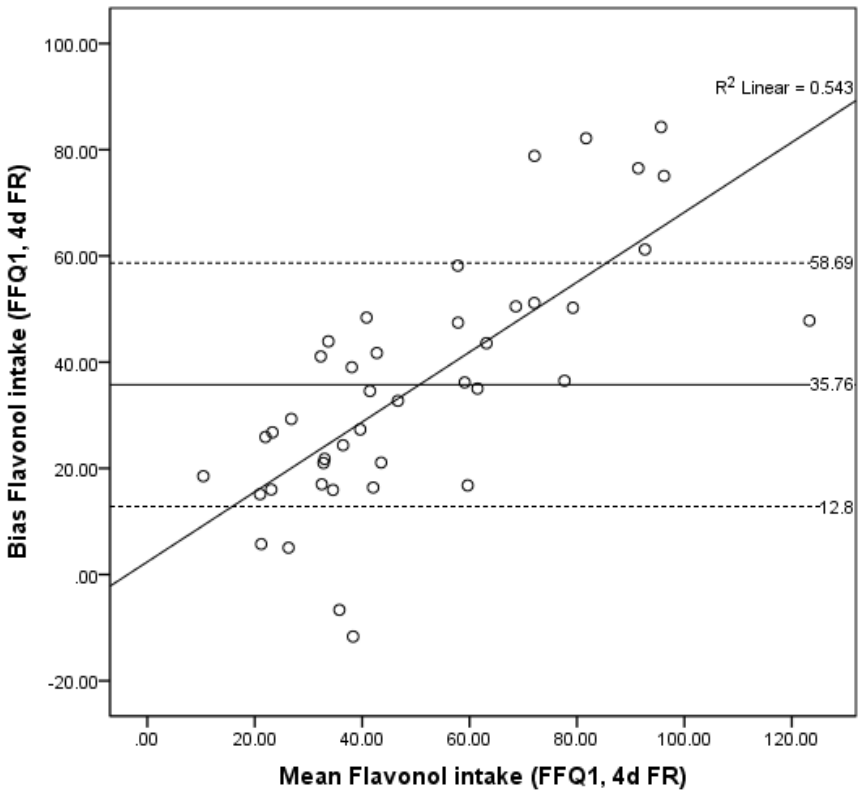
(c)


R2 Linear = 0.090
100.00
50.00
.00
-50.00
60.89
23.95
-12.99
.00
20.00
40.00
60.00
80.00
100.00
Bias Flavanone intake (FFQ1, 4d FR)
Mean Flavanone intake (FFQ1, 4d FR)

(d)



(e)



(f)

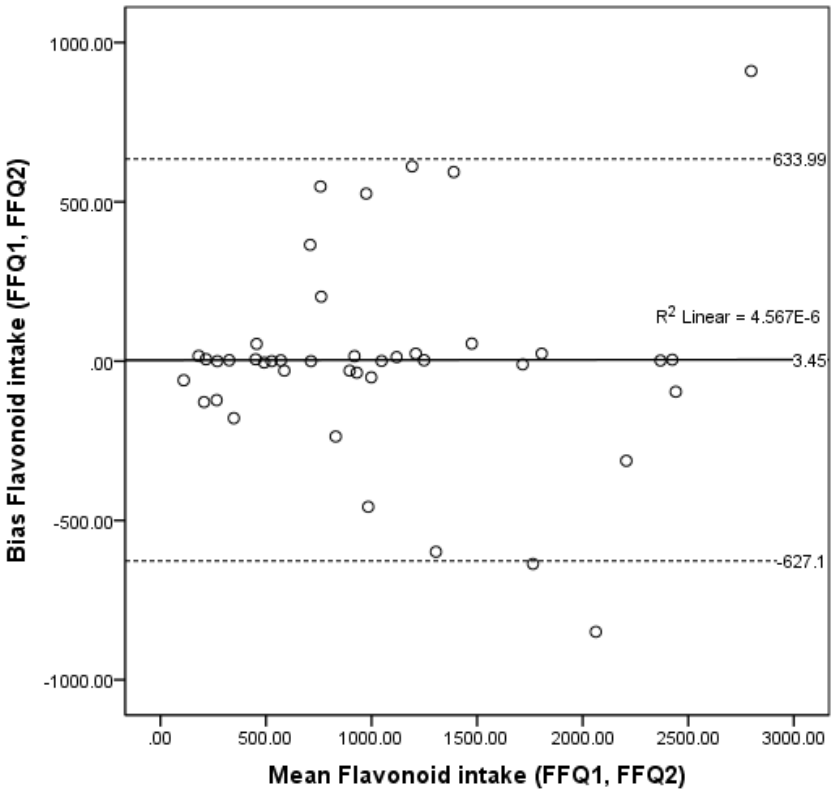
Figure 3-1 Bland Altman plots (difference in intake (mg/day) (FFQ1 – 4-day FR) against the mean intake of flavonoids and subclasses (mg/day) [FFQ1+4-day FR)/2]) showing the relative validity of the FFQ 1 vs the 4-day FR for total (a) flavonoids, (b) anthocyanin, (c) flavan-3-ol, (d) flavanone, (e) flavone and (f) flavonol intake.

3.3.2 Reproducibility

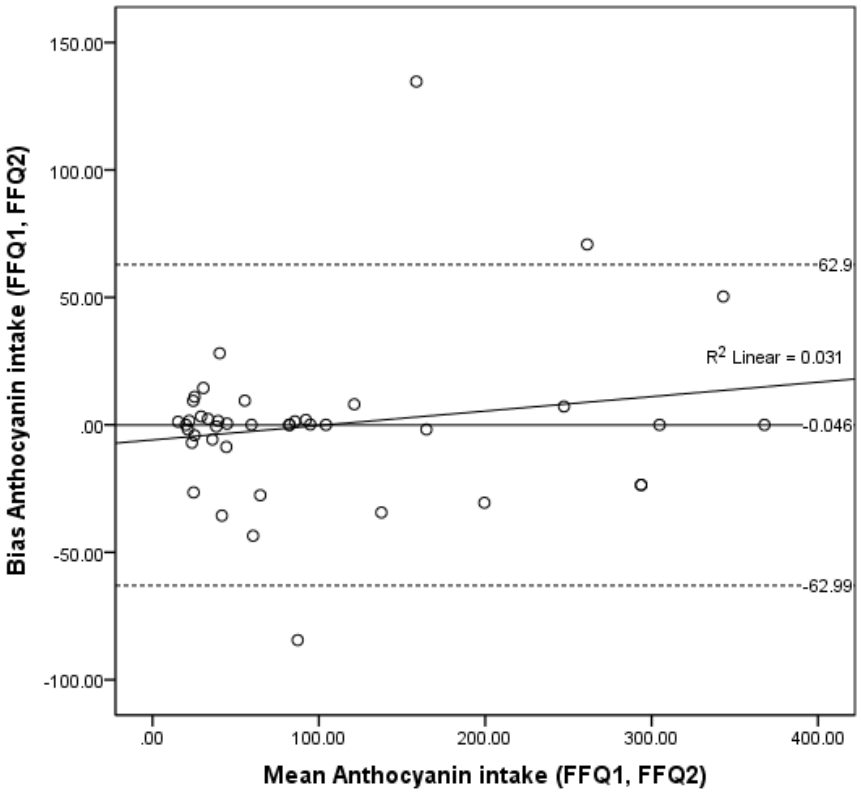
The mean percentage difference between FFQ1 vs FFQ2 showed small variations for total flavonoid intake (6.74%), and intake of anthocyanins (13.75%), flavan-3-ols (14.07%), flavanones (10.73%), flavonols (-1.11%), with slightly higher variation for flavones (22.34%).

Wilcoxon signed rank sum test showed no significant differences in the measurement of total flavonoid or any flavonoid subclass intakes (mg/day) between the FFQ1 and FFQ2 (Table 3-5). Total flavonoid intake showed excellent reliability (>0.9) and each flavonoid subclass showed between acceptable (>0.7) and excellent reliability (Table 3-5). The Bland Altman plots visually showed small, non-significant bias (Figure 3-2) and wide limits of agreement. Linear trends in the bias are not evident between FFQ1 and FFQ2 (Figure 3-2).

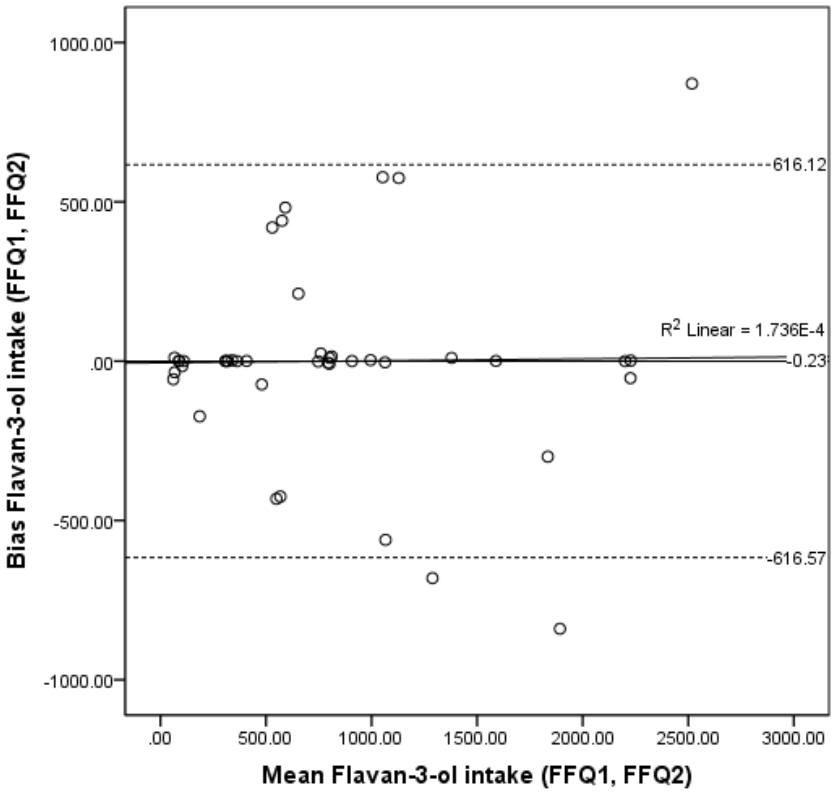
The Cohen's kappa (κ) indicated substantial agreement (21) between the FFQ1 and FFQ2 for total flavonoid intake ($\kappa = 0.619$, $p<0.001$), anthocyanin intake ($\kappa=0.651$, $p<0.001$), flavan-3-ol intake ($\kappa=0.746$, $p<0.001$), flavone intake ($\kappa=0.714$, $p<0.001$) and flavonol intake ($\kappa = 0.619$, $p<0.001$), and moderate agreement (21) for flavanone intake ($\kappa=0.492$, $p<0.001$).


Bias Flavonoid intake (FFQ1, FFQ2)
1000.00
500.00
.00
-500.00
-1000.00
633.99
3.45
-627.1
R2 Linear = 4.567E-6
.00
500.00
1000.00
1500.00
2000.00
2500.00
3000.00
Mean Flavonoid intake (FFQ1, FFQ2)

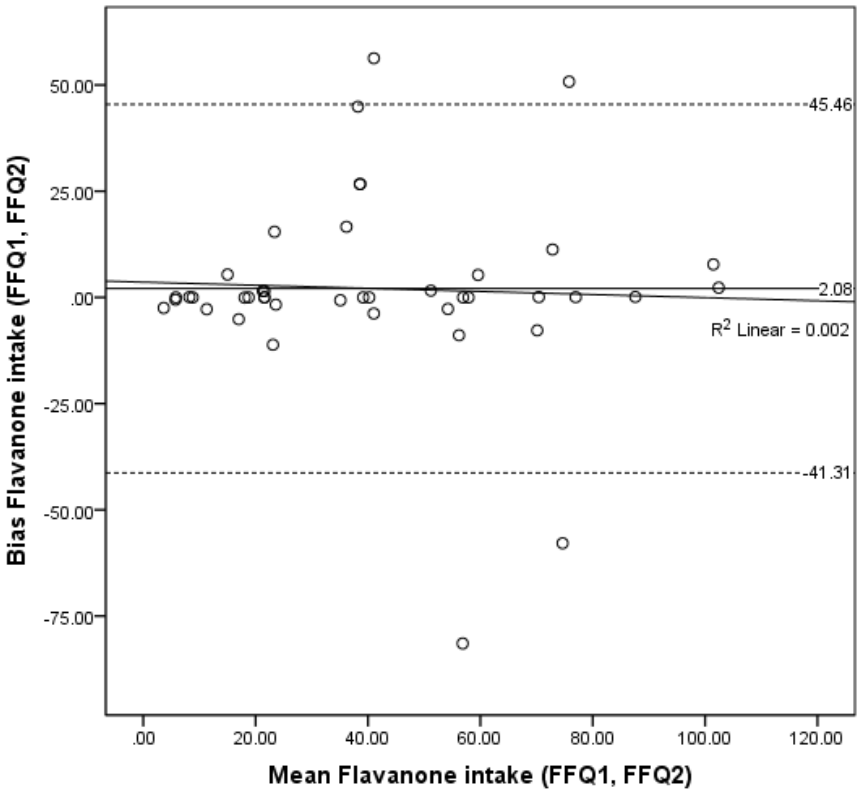
(a)


Bias Anthocyanin intake (FFQ1, FFQ2)
150.00
100.00
50.00
.00
-50.00
-100.00
62.9
-0.046
-62.99
R2 Linear = 0.031
.00
100.00
200.00
300.00
400.00
Mean Anthocyanin intake (FFQ1, FFQ2)

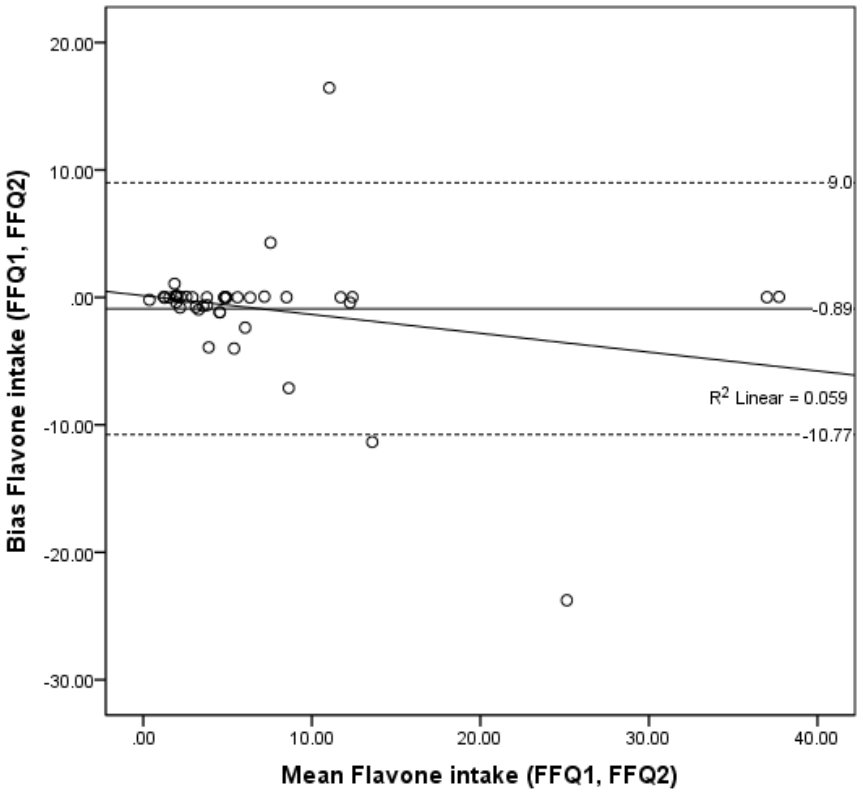
(b)


Bias Flavan-3-ol intake (FFQ1, FFQ2)
Mean Flavan-3-ol intake (FFQ1, FFQ2)
1000.00
500.00
.00
-500.00
-1000.00
616.12
-616.57
R2 Linear = 1.736E-4
.00
500.00
1000.00
1500.00
2000.00
2500.00
3000.00

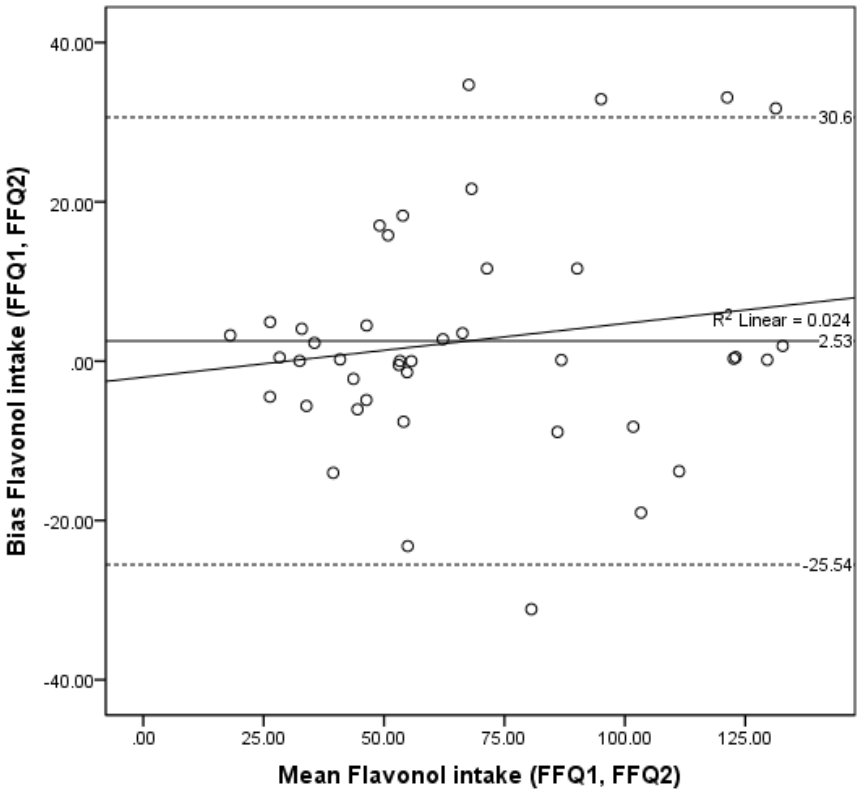
(c)


Bias Flavanone intake (FFQ1, FFQ2)
Mean Flavanone intake (FFQ1, FFQ2)
50.00
25.00
.00
-25.00
-50.00
-75.00
45.46
2.08
-41.31
R2 Linear = 0.002
.00
20.00
40.00
60.00
80.00
100.00
120.00

(d)



(e)



(f)

Figure 3-2 Bland Altman plots (difference in intake (mg/day) (FFQ1 – FFQ2) against the mean intake of flavonoids and subclasses (mg/day) [FFQ1+FFQ2)/2]) showing the relative validity of the FFQ 1 vs the FFQ2 for total (a) flavonoids, (b) anthocyanin, (c) flavan-3-ol, (d) flavanone, (e) flavone and (f) flavonol intake.

3.4 Discussion

A novel flavonoid-specific FFQ was developed for use in older Australians by applying a systematic approach using data obtained from 12 days of weighed FRs and the most appropriate flavonoid composition data (18). Total flavonoid intake as measured by the FFQ (1050mg/day) was significantly higher than that reported in other Australian studies (1, 4, 23), including the analysis of the cohort of older adults who were used to inform the development of this tool (14) (683mg/day). The 4-day FR similarly measured flavonoid intake (847mg/day) and intake of flavonoid subclasses as being higher than previous Australian estimations, however the percentage contributions of each flavonoid subclass to total flavonoid intake are closely related to previously reported estimates for the total Australian population (1). An overestimation of flavonoid intake was similarly reported in a validation study of an FFQ developed to measure flavonoid intake in younger Australian adults (9), and appears to be a typical finding when validating a FFQ against FRs (24). Nevertheless, the major sources of total flavonoids as estimated by the FFQ are black tea (57.0%), red wine (7.6%) and green tea (6.0%), which has previously been described as significant flavonoid sources in this age group (1). Inclusion of both the most commonly consumed flavonoid-containing foods; in addition to the richest dietary sources according to the food composition database in the FFQ provided a sound basis that enabled the most accurate estimation of habitual intake of all flavonoids. For example, although consumed sporadically and in small amounts, parsley provided the major source of flavone intake. Previous dietary questionnaires that have not incorporated both considerations (10, 25, 26) and have shown limitations in the assessment of total flavonoid intake.

Despite the FFQ1 and 4-day FR being strongly correlated, except for flavanone and flavone intake, there were significant differences in estimation of the total flavonoid intake and intake of flavonoid subclasses (except for flavan-3-ols) between the two measures. This is a result of the FFQ consistently measuring flavonoid intake to be higher than the 4-day FR analyses. This finding was confirmed by the Bland-Altman analysis, which indicates a case of proportional error (strong positive correlation) (Figure 3-1). As correlation quantifies the degree to which two variables are related, but does not automatically imply that there is good agreement between the two methods (20), this finding is not uncommon. The agreement between the FFQ and the 4-day FR to assign an individual to quartiles of intake suggests that the FFQ is an excellent method for total flavonoid intake, but exhibits differing levels of agreement for

individual subclasses of flavonoid, particularly flavanone intake which are commonly found in citrus related foods. The misclassification for the flavonoid subclasses may also relate to the influence of seasonal foods being under-reported in the 4-day FR in comparison to the FFQ. This is of particular relevance for anthocyanins and flavanones that showed the poorest agreement between methods. Berries and cherries (summer fruits) and mandarins (winter fruit) are highly seasonal fruits that are available for short periods of time in Australia, but are rich in anthocyanins and flavanones, respectively. The period of reporting for the FFQ was consumption of fruits and vegetables over the previous 12 months, whereas the 4 day-FR measured intake over a short period between June and September which corresponds to winter and early spring in Australia. Thus, the FFQ could be expected to over-estimate the flavonoid intake related to seasonal fruit consumption. Alternatively the 4-day FR, which was considered to be robust reference method in our study, could be considered inadequate at estimating overall habitual flavonoid intake. These considerations are important when deciding on the purpose of the instrument, whether to measure absolute intakes in order to assess acute changes as would be needed in a short term intervention study or clinical trial, or whether the instrument is required for ranking individuals according to usual intake, as is required in epidemiological studies (12). Our study has demonstrated the latter, as is the desirable strength of a FFQ and highlights its potential application for use in large cohort studies.

Our findings are consistent with a Flemish study in which an 86-item FFQ was validated against a 4-day non-consecutive food diary in dietitians (27). That FFQ was similarly able to assign subjects to correct quartiles of flavonoid intake and to identify high flavonoid containing foods and regular sources of flavonoid intake, but was less suitable for estimating total intake (mg/day). The FFQ also showed only weak or no correlation to the 4-day food diary in relation to specific flavonoid subclasses, for example, anthocyanins (27). In that study, the poor correlation was related to the sporadic consumption of high flavonoid-containing foods, such as red wine, that were not captured in the 4-day food diary; a result which appears to be replicated in our study.

Using various statistical tests, the novel FFQ developed by this study showed an excellent level of reproducibility. The repeated FFQs were highly correlated, displayed a small percentage disagreement (21), and showed no difference between measured total intakes of flavonoids or their subclasses. These findings were confirmed in the Bland-Altman analyses that visually

indicated a low level of bias and the Cohen's kappa (κ) results that indicated moderate (κ = 0.41– 0.60) to substantial agreement (κ = 0.61–0.80) (21) between the two time points. The FFQs were administered one month apart; therefore it is unlikely that any seasonal variation in diet would have impacted its reproducibility.

There are limitations to this study, which partially relate to the general limitations associated with using a FFQ to measure dietary components. While FFQs are a cheap and relatively simple to apply, they are associated with large measurement error due to inaccuracies in estimating frequencies and food serving sizes (12). Longer FFQs have shown to overestimate fruit and vegetable intake (12), which could have been a contributor to measurement error in this study. Another potential source of bias in validation studies of this nature is that, because the FFQ was unable to make provision for information on the cooking processes associated with each food, a 'raw food' value was attributed for each food which may have over-inflated intake values. However, in the case of the 4-day FR method cooking-related flavonoid losses were able to be accounted for as cooking methods were advised. Future work should attempt to address the amount of cooked vs fresh food consumption that is associated with of each food item. The potential to add a retention factor to the raw food values and address the degradation associated with cooking should be investigated in the further refinement of this instrument. Future research should also consider the influence of the reference food composition database used, and the impact that it may have on the estimated flavonoid intake. A recent study by Witowska et al., (28) showed that utilizing the Phenol Explorer database and the USDA database resulted in different polyphenol estimations in Polish adults. However, until there is an Australian specific, comprehensive flavonoid reference database, then the relative validity of applying one database versus another (i.e. USDA vs Phenol Explorer) is questionable.

To improve the validity of a FFQ, design issues including the length, closed vs open-ended responses, seasonality, time-frame and portion sizes can be manipulated (12). Future research may focus on adjusting the time-frame of the recall if short-term intake is required over habitual consumption. Additionally, a shorter version of the FFQ could be trialled, excluding foods that did not significantly contribute to flavonoid intake in our study (e.g. herbs). The usefulness of this FFQ over other general FFQs, such as the Block, Willett, and National Cancer Institute FFQ (16), should be a consideration for future research. However, due to the highly specific flavonoid content of foods, grouping of food items that are similar nutritionally (e.g. citrus

fruits) into a single question (12), which is the case for many pre-existing FFQs (16), is not recommended. Lastly, as diet is highly influenced by season, future studies may consider confirming these findings in all seasons across a year.

3.4.1 Conclusion

A novel FFQ developed to estimate flavonoid intake for use in older Australians has been shown to demonstrate fair validity against a 4 day FR method and substantial reproducibility. The FFQ could allow for an easier estimation of flavonoid intake and intake of flavonoid subclasses in older adults, especially when ranking individuals according to total flavonoid intake in epidemiological research. Future studies may attempt to validate this tool against repeated FRs collected across several seasons, in order to assess the instrument's ability to more accurately capture seasonal food intake, and potentially improve the validity of the tool for measuring flavonoid subclasses.

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CHAPTER 4: DEVELOPMENT OF A GAS CHROMATOGRPHY/MASS SPECTROMETRY TECHNIQUE TO DETERMINE PHENOLIC ACID BIOMARKERS OF FLAVONOID INTAKE AND METABOLISM

This chapter describes the potential for phenolic acid metabolites as biomarkers of flavonoid intake. This chapter demonstrates the candidate's knowledge about the analytical techniques of Gas Chromatography (GC) and Mass Spectrometry (MS). It the describes the process undertaken to develop and establish a GC-MS technique to measure phenolic acids in human plasma at the University of Wollongong, in the Illawarra Health and Medical Research Institute under the supervision of Dr. Andrew Jenner (Senior Research Fellow). This method is applied in Chapter 6 of this thesis: 'Developing methodology to assess the acute dose-dependent effects of flavonoids in cherry juice on cognition and blood pressure: a pilot cross-over study.'

KK carried out experimental work and wrote the manuscript. Dr A Jenner devised and supervised the experimental work. Dr A. Jenner is an experienced GC/MS scientist, who trained KK in GC/MS methodology, carried out the GC/MS maintenance and designed the GC/MS programs. Undergraduate students, Linnea Svenson and Vijay Kuppurajan also contributed to the experimental work related to the method development.

4

Background

4.1 Flavonoid Absorption and Metabolism

Flavonoids encompass a wide group of compounds that form a subclass of polyphenols and comprise 15 carbons, with two aromatic rings bound together by three carbon atoms (denoted as A, B and C) that form an oxygenated heterocycle (Figure 4-1). Common flavonoids and their chemical structure are shown in Table 4-1.

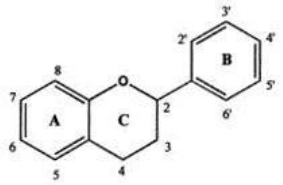
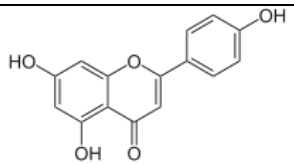
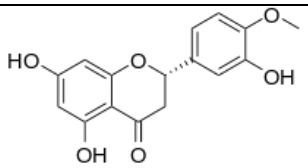
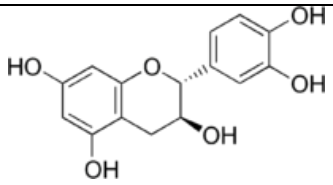
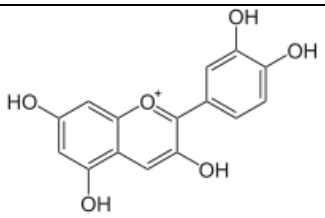
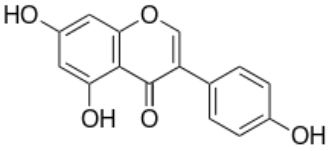
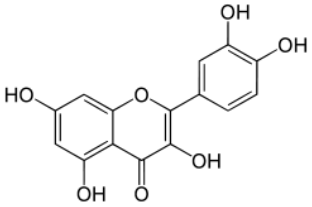


Figure 4-1 Generic flavonoid structure (1)

Until relatively recently, very little was understood regarding flavonoid bioavailability, absorption and metabolism. However, with evidence from human studies linking flavonoid intake to improved health outcomes, there has been a rapid increase in our knowledge surrounding their metabolism and bioactive effects (2). The bioavailability of specific flavonoids that are abundant in the human diet has been well established and reviewed (3). Manach et al., 2005 (3) reviewed 97 studies to conclude that polyphenols (including flavonoids) markedly differ to one another in relation to their bioavailability and intestinal metabolism. Additionally, intra-individual variation significantly impacts their uptake and biological effects (4).

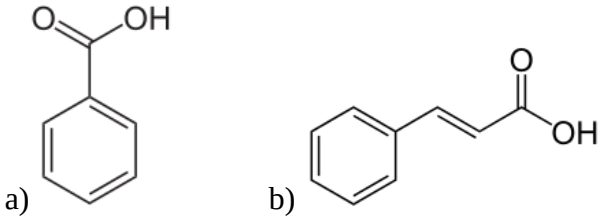
Table 4-1 Chemical structure of common dietary flavonoids

Flavonoid group	Example	Chemical Structure
Flavones	Apigenin	
Flavanones	Hesperetin	
Flavan-3-ols	Catechin	
Anthocyanins	Cyanidin	
Isoflavones	Genistein	
Flavonols	Quercetin	

Flavonoids are subjected to extensive metabolism following ingestion. Flavonoid metabolism may begin in the oral cavity, with hydrolysis of flavonoid glucosides occurring when they make contact with saliva, which possesses strong hydrolic activity (5). In the upper GI tract, evidence suggests that only a small proportion of dietary flavonoids are directly absorbed and become available to tissues and cells (1-2%). Some flavonoids may be absorbed through the gut and once absorbed, they are transported to the liver where they are conjugated, commonly by methylation, sulphation and glucuronidation (6). Secretion via the biliary route will expose the conjugated compounds to the gut flora and are therefore subjected to reabsorption in the colon (6). A significantly greater proportion of dietary flavonoids are passed through the intestine to

the colon, where an abundance of colonic microflora metabolise them into a variety of phenolic acids and other metabolites (3).

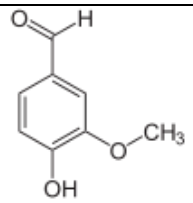
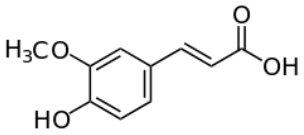
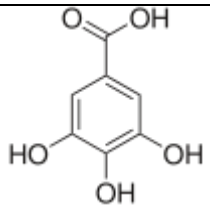
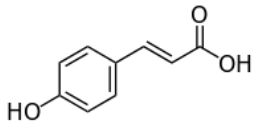
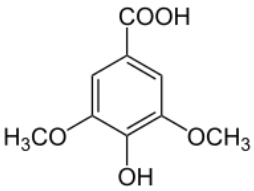
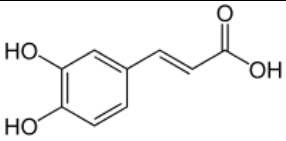
The structures of phenolic acids are related to derivatives of either benzoic or cinnamic acids (Figure 4-2), with some common phenolic acid metabolites and their structure is shown in Table 4-2. Although the basic skeleton remains the same, the numbers and positions of the hydroxyl groups on the aromatic ring produces is responsible for the many different phenolic acids (1). Phenolic acids are consumed through their presence in all plant-based foods, in addition to their endogenous production via the metabolism of flavonoids.



a) b)

Figure 4-2 a) Benzoic Acid Chemical Structure b) Cinnamic Acid Chemical Structure (adapted from (1))

Table 4-2 Common phenolic acid metabolites according to Benzoic or Cinnamic Acid derivatives

Benzoic Acid Derivatives	**Structure**	**Cinnamic Acid Derivatives**	**Structure**
Vanillin		Ferulic Acid	
Gallic Acid		p-Coumaric Acid	
Syringic Acid		Caffeic Acid	

It has only been fairly recently that the biological actions of flavonoid metabolites, including phenolic acids, has been investigated, meaning their relative contributions to the health benefits of a flavonoid rich diet are largely unknown. However, the association of phenolic acids and health outcomes has been made, and is largely underpinned by their antioxidant, anti-inflammatory and anti-proliferative activities (1, 7). Given the vast difference in bioavailabilities between flavonoids and phenolic acids, it has been hypothesised that phenolic acids may therefore be responsible for the observed health effects of flavonoid consumption.

4.2 Biomarker analysis

Biomarkers are important in the study of the effects of diet on health outcomes. It is usually defined as any biochemical measure of events occurring in a biological system following dietary exposure to a particular compound (8).

Dietary flavonoid intake can be determined by quantifying biomarkers which include intact flavonoids and their derivatives (e.g. phenolic acids) found in plasma, urine and faecal water (9). Many methods of measuring flavonoid biomarkers in human biological samples exist, with no standardised protocol of how to perform this analysis. Consequently researchers must develop and validate their own methods, limiting the ability to compare studies that have used different methods to measure certain biomarkers. As there many thousands of flavonoids identified, that are quickly and extensively metabolised into various metabolites, there are thousands of potential biomarkers (10). To date, there is no consensus around which flavonoids or metabolites are indicative of total dietary intake.

In addition, it is currently unknown which biological sample (plasma, urine or faecal water) should be selected and some research suggests each maybe indicative of different consumption patterns. Previous research shows urinary biomarkers may be more reflective of short-term intake (11). The flavonoid content in fasting plasma or faecal water samples seems to be a suitable biomarker of short-term intake and a possible biomarker of the medium-term intake (11). However, biomarkers of long-term intake are not yet identified and may be unlikely due to the short half-lives of dietary flavonoids *in vivo*. Most of the biomarker analyses are expensive and quite often it is not possible to perform them as part of a large epidemiological study (12).

4.2.1 Flavonoids as biomarkers

Measurement of intact flavonoids (generally in a glucuronidated form) provides a potential biomarker to assess the intake and biological activities of flavonoid rich foods and can be measured in urine and plasma. There is little indication of the expected concentrations of flavonoids in biological samples, with examples from current literature highlighted in table 4-3. Unfortunately, there are few expected flavonoid concentrations in plasma published in the

literature. With such a small and variable percentage of flavonoids consumed reaching the circulatory system intact (Table 4-3), they may be difficult to measure.

Table 4-3 Concentrations of a selection of flavonoids in human plasma (fasting)

	Phenolic compounds	**Plasma concentration (nM) Mean±SD**
Radtke et al., 2002 (11)	Quercetin	22.87±16.61
	Kaempferol	10.65±7.89
	Naringenin	8.15±15.43
	Hesperetin	22.16±44.85
Cao et al., 2010 (13)	Quercetin	80.23±81.73
	Kaempferol	57.86±60.88
	Isorhamnetin	39.94±45.01
	Apigenin	10.62±12.26
	Luteolin	99.9±97.65

4.2.2 Phenolic acid metabolites as biomarkers

Phenolic acids can also be measured as metabolic biomarkers of flavonoid intake and can be quantified in plasma (14), urine (14) and faecal water (15). Table 4-4 shows the concentrations of some phenolic acids in human plasma, measured using a GC-MS technique.

Due to the complexity of plant-based foods, which contain many hundreds of compounds, the best metabolite for each flavonoid or flavonoid containing food, and which is indicative of dietary intake or reflects the bioactive effects, remains unknown (3, 10). Thus, a ‘metabolite profiling’ approach, in which researcher’s measure multiple biomarkers related to flavonoid intake, may be a superior method a single biomarker (flavonoid or phenolic acid) to improve the specificity of dietary exposure to a compound or food (4, 14).

Table 4-4 Concentrations of a selection of phenolic compounds in human plasma

Phenolic compounds	**Plasma concentration (nM) Mean±SEM (range)**
4 ethyl phenol	1749 ± 233 (387, 8713)
phenyl acetic acid	49411 ± 2559(13160, 97114)
Catechol	1306 ± 631 (93, 30835)
3-phenylpropionic acid	682.20±117.19 (192.78, 5504.99)
t-cinnamic acid	173.20±22.31 (0.00, 640.21)
3-hydroxyphenylacetic acid	356.51±82.64 (0.00, 2401.27)
4-hydroxybenzoic acid	2511.89±101.00(1403.93, 4211.34)
4-methoxyphenylpropionic acid	545.40±77.54 (0.00, 1922.35)
4-hydroxy 3-methoxybenzoic acid (vanillic acid)	262.83±15.18 (98.72, 465.94)
2-hydroxycinnamic acid (*o*-coumaric acid)	590.99±127.40 (0.00, 3417.39)
3,4-dihydroxybenzoic acid	227.51±18.75 (73.10, 430.01)
3,4-dihydroxyphenylacetic acid	7.89±2.09 (0.00, 49.68)
3,4,5-trihydroxybenzoic acid (gallic acid)	5.05±1.73 (0.00, 58.35)
4-hydroxy 3-methoxycinnamic acid (ferulic acid)	40.12±6.85 (0.00, 193.38)
3,4-dihydroxycinnamic acid (caffeic acid)	1707.24±595.77 (71.87, 18887.12)

Values via Dr A Jenner. Personal Communication, 14 April 2011

The rapid and systematic measurement of phenolic acids and flavonoids in biological samples is challenging, due to their inherent structural diversity and low concentrations in biological samples. A recent review highlighted the numerous and various was in which researchers have quantified phenolic acids and flavonoids in samples (both biological and plant-based) (1). They concluded that the analytical methods selected by researcher depend on a variety of factors including the availability of tools and techniques, the sensitivity needed and the complexity of the biological matrix (1, 9). Therefore, generally a combination of a chromatography technique with sensitive detection method is utilised.

4.3 Gas Chromatography-Mass Spectrometry

4.3.1 Gas Chromatography

Various types of chromatography can be used in biomarker analysis (9, 16). The methodology related to Gas Chromatography (GC) will be discussed in more detail. A GC system consists of carrier gas, an injector, a column (with a stationary phase) and a detector (16) (Figure 4-3).

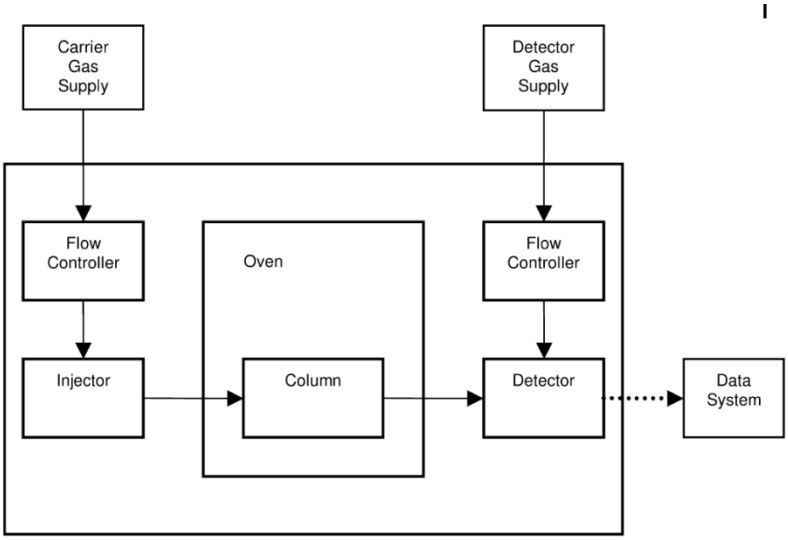


Figure 4-3 Gas Chromatography system diagram (17)

4.3.1.1 Sample preparation

Generally, an analyte may have to undergo derivatization to become more suitable for GC analysis through making compounds more volatile or less polar, improving thermal stability and introducing a marker on the compound that can be detected (18). The derivatization reaction commonly occurs by alkylation, silylation, and acylation of polar functional groups.

4.3.1.2 Injection

The injection of a sample into the GC column involves rapid vaporisation of a liquid sample (0.2 – 3µL) and subsequent mixing with a carrier gas, before injection via an inlet into the GC column (17). The inlet is heated (to up to 400º Celsius) to ensure that the entire sample evaporates quickly and mixes with the carrier gas. Carrier gases (mobile phase) are usually very pure (<99.99%) sources of helium, hydrogen, nitrogen or argon, which carry the sample through a GC column where it is separated into individual components.

4.3.1.3 Column separation

The column (comprising tubing and a stationary phase) sits in a temperature controlled oven that is able to separate compounds based on their solubility and boiling point. As the sample reaches the column, it will cool and condense, moving in a tight band around the GC column (20). The analytes that are lighter and have lower boiling points will move more quickly around the column towards the detector. The oven temperature is pre-determined to rise in a tightly controlled temperature program, which is established to manipulate the speed at which compounds will be fed through the column, to enhance separation of the compounds of interest (20).

4.3.1.4 Detection

After the separation of the analytes that are eluted from the column, they are individually measured in a detector that converts a chemical or physical property into an electrical signal (21). A PC based data system is used to process the signal from the GC to determine peak areas: peak heights and widths and retention times. The chromatogram, which is the graphical representation of the sample elution, is displayed. The height of the peak or the area under the peak is used to determine the amount of the compound in the original mixture. Each peak is displayed by the exact time it leaves the column and passes to the detector (retention time).The retention time provides a qualitative measure of the compound, which can determine the presence of a certain compound, by comparing the sample peak times with known reference standard peaks times. Analysis of the peak area or peak height quantitates the amount of the sample component. Standard amounts of the compounds can be used as a reference to make a calibration curve to produce quantitative results for each calibration peak. Comparison of the peaks produced by a sample to the calibration curve indicates the amount of the compound of interest in the sample mix.

4.4 Mass spectrometry

Mass spectrometry (MS) is an analytical quantification technique designed to identify molecules by measuring their mass-to-charge ratio (*m/z*). MS is highly regarded as a sensitive

and quick analytical method, with a particular strength in application for biochemical compound analysis (22). The principle of this technique is to ionize the molecules and create smaller fragments, which can then be identified by molecular mass (21). The three essential components of a mass spectrometer are the ion source, which converts the molecules into ions; a mass analyser, which sorts the ions depending on their mass and charge and finally; a detector that quantifies the amount of each ion (21) (Figure 4-4).

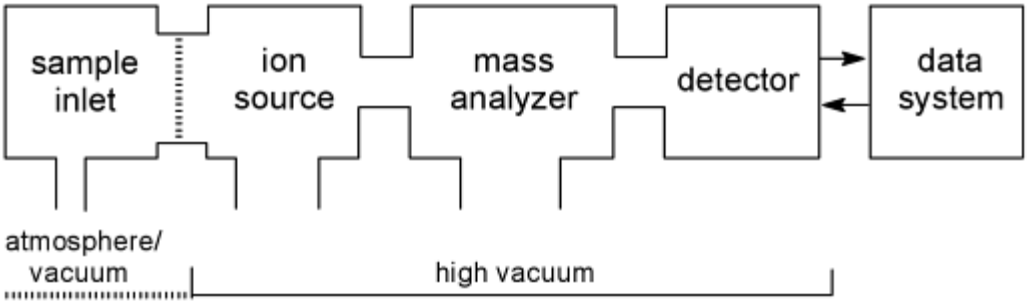


Figure 4-4 Schematic diagram of a Mass Spectrometer (23)

4.4.1.1 Triple Quadrupole Mass Spectrometer

In a triple quadrupole mass analyser is a tandem mass spectrometer consisting of two quadrupole mass analysed with a collision chamber between them. Essentially, the triple quadrupole mass spectrometers operate under the same pretence as the single quadrupole mass analyser. However, a triple quadrupole mass analyser has the advantage of being more sensitive and is able to detect smaller low energy low molecule reactions, which is useful when small molecules are being analysed.

4.4.1.2 Ionisation

Ionisation is the first step in MS analysis, which acts to produce gas-phase ions of the compound of interest. In the ionisation source, the sample molecule is bombarded with a high-energy electron beam to form a radical cation (also called a parent/precursor ion). The process leaves the molecule with an excess of energy, leading to instability and fragmentation of the molecule into various pieces (product ions). Fragmentation provides a unique 'fingerprint' for that molecular structure, resulting in ion fragments with a certain mass and charge *(m/z)*. Many

impacts occur simultaneously, generating numerous different ions and fragments for each compound.

4.4.1.3 Mass Filtration/Mass Analyser

The positive ions which are formed in the ioniser, are accelerated down a curved tube (deflected by a magnet) into the mass analyser where they are separated in space, and are then identified based on their mass/charge ratio (21). Lighter ions will be deflected to a greater extent than heavier ions and a 'mass spectrum' is created by the different mass ions that are detected.

4.4.1.4 Detection

After the ions are separated they enter a detector and a 'mass spectrum' is produced. The x-axis represents the *m/z* ratios. The y-axis represents the signal intensity (abundance) for each of the fragments detected during the scan. A computer database of mass spectra can be used to match compounds in an unknown sample.

4.4.2 Benefits of combining GC with MS for measuring phenolic acids in biological samples

The combined GC-MS procedure is very useful when dealing with a sample that is a mixture of two or more different compounds, as the various compounds are separated from one another before being subjected individually to MS analysis. Combining GC and MS have great advantages when analysing biological metabolites due to its high sensitivity and specificity. High specificity (24) is desirable in an analytical method to accurately positively identify the presence of a particular substance. A high sensitivity is necessary in assessments where the biological concentrations are low, and only a small amount of biological is sample needed for a reliable quantitative analysis (22). Additionally, there are extensive compound databases and experimental protocol available to researchers developing this technique (22). Less favourable attributes of GC-MS includes the need to derivatize samples to improve their volatility and thermal stability before injection, which is labour-intensive and may produce derivatives that will obstruct the quantification (9, 25).

While many analytical investigations into phenolic acids and flavonoids have been reported (9), there is no gold standard method for their separation and quantification in biological samples, leaving researchers to develop their own techniques.

4.5 Aim

The aim of this work was to develop a sensitive method for analysing various phenolic acids and flavonoids in biological samples using a standardised method with a GC-MS triple quadrupole mass analyser.

The objectives of the study were to:

1. Develop a library of spectra (database) to provide a reference for the identification of compounds in future analysis;
2. Optimise the programs for the GC and MS portions of the analysis for the separation and detection of phenolic acids and flavonoids.

4.6 Methods

4.6.1 Materials

The instrument used was an Aligent 7000 GC/MS-Triple Quad, stationed at University of Wollongong, Australia. The separation was carried out on 30 m long capillary column with a diameter of 0.25 mm. The stationary phase was made up of a 0.25µM thick 5% phenyl methylpolysiloxane film and 1 µl was injected through a splitless inlet. Helium was used as carrier gas with a flow rate at 1.2 ml/min. Positive chemical ionisation (PCI) was used and the reagent gas in the ionisation chamber was methane. Argon gas was used in the collision chamber in the mass analyser.

4.6.2 Preparation of standards for database

To create an in-house library of spectra, injections of pure standards need to be processed for reference. For an adequate database, all patterns of the retention times of compounds and the mass spectrum of the pure standard need to be recorded.

Reference compounds of flavonoids and phenolic acids (full list in Table 4-5) were dissolved in an appropriate medium (acetonitrile, methanol or isopropanol) and made up to a concentration of 25mM. Seven internal standards were used for quantification (d5-benzoic acid; phenyl-d7 acetic acid; 3-phenyl-d9 propionic acid; *t*-cinnamic acid (phenyl-d7); 2-OH,6-

MeO benzoic acid; 2,4,5-triMeO cinnamic acid and 6-methylflavone) and were prepared in the same way. Internal standards are added in a constant amount to the samples, the blank and the calibration standards and are used to account for the loss of analyte during processing.

Each compound was added to individual vials and dried off in a heater, before adding 10 µl of acetonitrile and 30 µl of derivatizing agent *N*,*O*-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA). The samples were incubated for five hours before injecting to the GC-MS. Assessment of retention times was conducted and the mass spectrum for each compound was determined. As a compound's spectrum is very reliable and reproducible, the instrument can search the database and identify each compound in the sample, based on similarities within the database.

4.7 Results

4.7.1 Assessment of retention times

The first step in this process is o optimise the separation of the compounds using GC. A mixture of all compounds was made and analysed with various temperature programs designed to improve the separation of compounds, so each compound elutes at a different time. Trial-and-error was used to optimise the separation. The initial temperature programs tested were based on the method Jenner, Rafter and Halliwell (2005) (15), with some adjustments, resulting in retention times (in minutes) as per Table 4-5. An example of the output for the elution from the GC for Cinnamic Acid at 8.6minutes is shown in Figure 4-5.

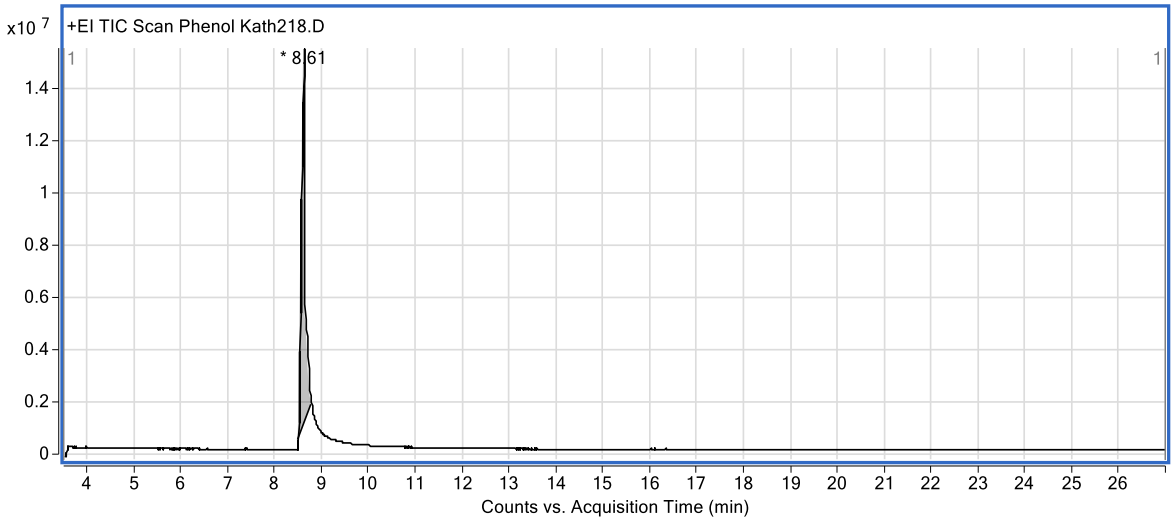


Figure 4-5 Elution time for Cinnamic Acid (8.6m)

Table 4-5 The 115 reference compounds, retention times and selected transitions comprising the in-house database

Compound	Retention Time (m)	Transition
4-ethyl phenol	3.47	194.1 -> 179.1
benzoic acid - d5	3.55	184.1 -> 110.1
benzoic acid	3.57	179.1 -> 105.1
catechol (1,4-diOH benzene)	3.78	254.2 -> 239.2
phenyl acetic acid - d7	4.2	200.1 -> 172.1
phenyl acetic acid	4.26	193.1 -> 165.1
1,3,5-triMeO benzene	5.9	168.1 -> 139.1
3-phenylpropionic acid - d9	6.1	231.2 -> 112.1
3-phenylpropionic acid	6.2	222.2 -> 104.1
4 ethyl benzoic acid	6.95	207.1 -> 133.1
2 MeO benzoic acid	7.05	209.1 -> 135.0
3,4-diMeO phenol	7.1	226.1 -> 221.0
1,2-diOH, 3-MeO benzene	7.15	284.1 -> 269.0
1,4-diOH, 3-MeO benzene (3-MeO catechol)	7.2	284.1 -> 269.0
3-MeO benzoic acid	7.3	209.1 -> 135.0
2-MeO phenyl acetic acid	7.73	238.1 -> 179.1
3,5-diMeO benzene	7.85	226.1 -> 211.0
2-OH benzoic acid	7.9	209.1 -> 165.1
4-MeO benzoic acid	8	209.1 -> 165.1
2-MeO phenyl acetic acid	8.3	238.1 -> 194.1
4-OH, 3-MeO benzaldehyde (vanillin)	8.49	224.1 -> 209.1
t-cinnamic -d7	8.55	212.1 -> 168.1
t-cinnamic acid	8.6	205.1 -> 161.1
4-MeO phenyl acetic acid	8.63	238.1 -> 194.1
pyrogallol (1,2,4 tri-OH benzene)	8.7	239.1 -> 211.1
3-OH benzoic acid	9.1	267.1 -> 223.1
2,3-diMeO benzoic acid	9.6	239.1 -> 224.1
2-MeO phenylpropionic acid	9.75	252.1 -> 134.1
2-OH phenylacetic acid	9.8	296.1 -> 252.1
3-OH phenyl acetic acid	9.9	296.1 -> 252.1
3,4-diOH benzaldehyde	9.95	282.1 -> 267.1
4-OH benzoic acid - d4	10.32	271.1 -> 227.1
4-OH benzoic acid	10.38	267.1 -> 223.1
3-MeO phenylpropionic acid	10.46	252.1 -> 134.1
2,6-diMeO benzoic acid	10.48	254.1 -> 239.1
4-OH phenyl acetic acid	10.58	296.1 -> 252.1
4-OH phenyl-2-propionic acid	10.7	310.1 -> 193.1
1,3,5-triOH benzene (phloroglucinol)	10.87	342.1 -> 327.1
4-MeO phenylpropionic acid	10.92	252.1 -> 134.1
2,5-diMeO benzoic acid	11.1	254.1 -> 224.1

2-OH,6-MeO benzoic acid (IS1)	11.75	297.1 -> 249.1
3,5-diMeO benzoic acid	11.85	254.1 -> 239.1
2,OH,5-MeO benzoic acid	11.9	297.1 -> 249.1
3,4-diMeO benzoic acid	12.1	239.1 -> 195.1
3,4-diMeO phenylacetic acid	12.2	268.1 -> 268.1
3-OH phenylpropionic acid	12.35	310.1 -> 192.1
2,3-diOH benzoic acid	12.8	355.1 -> 249.1
3,4,5-triMeO benzoic acid	13	284.2 -> 269.1
1,3,5 triMeO benzoic acid	13.1	284.2 -> 269.1
3-OH,4-MeO benzoic acid (isovanillic acid)	13.15	312.1 -> 297.1
2,6-diOH benzoic acid	13.17	355.1 -> 249.1
4-OH phenyl propionic acid	13.2	310.1 -> 192.1
4-OH,3-MeO benzoic acid (vanillic acid)	13.3	282.1 -> 267.1
2,3,4-triMeO benzoic acid	13.31	284.1 -> 269.1
(H) homovanillic acid-d3	13.32	270.1 -> 179.1
4-OH,3-MeO phenylacetic acid (homovanillic acid)	13.34	267.1 -> 179.1
2,5-diOH benzoic acid	13.45	355.1 -> 297.1
2-OH cinnamic acid (o-coumaric acid)	13.98	293.2 -> 147.1
3-MeO cinnamic acid	14.05	235.1 -> 191.1
3,5-diOH benzoic acid	14.25	370.1 -> 355.1
3,4-diOH benzoic acid	14.32	370.1 -> 193.1
2,4-diOH benzoic acid	14.35	355.1 -> 281.1
4-MeO cinnamic acid	14.36	235.1 -> 191.1
3,4-diOH phenylacetic acid	14.45	369.1 -> 179.1
4,5-O-diMe gallic acid	14.5	312.1 -> 297.1
Shikimic acid	14.6	357.1 -> 147.1
3,4,5-triMeO phenylacetic acid	14.7	298.1 -> 239.1
3,4-O-diMe gallic acid	14.9	312.1 -> 297.1
3-OH cinnamic acid	15	308.2 -> 293.2
2,4,5-triMeO benzoic acid	15.08	284.2 -> 269.1
4-OH,3-MeO phenylpropionic acid	15.16	310.1 -> 192.1
4-O-Me gallic acid	15.2	370.1 -> 355.1
3,5-O-diMe gallic acid (syringic acid)	15.4	312.1 -> 297.1
2,3,4-triOH benzoic acid	15.65	443.1 -> 281.1
4-OH cinnamic acid (p-coumaric acid)	15.76	293.1 -> 249.1
3-O-methyl gallic acid	15.84	400.1 -> 223.1
3,4-diOH phenyl propionic acid	15.85	412.1 -> 412.1
3,4,5-triMeO cinnamic acid	15.9	310.1 -> 295.1
3,4,5-triMeO phenylpropionic acid	15.95	312.1 -> 194.1
3,4,5-triOH benzoic acid (gallic acid)	16.03	458.1 -> 281.1
(H) 3,4-diMeO cinnamic acid-13C3	16.4	283.1 -> 268.1
3,4-diMeO cinnamic acid	16.45	280.1 -> 265.1
3,5-diMeO cinnamic acid	16.5	280.1 -> 265.1

4-OH,3-MeO cinnamic acid (ferulic acid)	16.85	308.1 -> 293.1
3-OH,4-MeO cinnamic acid (Isoferulic acid)	16.95	308.1 -> 293.1
3,4-diOH cinnamic acid (caffeic acid)	17.26	396.1 -> 219.1
2,4,5-triMeO cinnamic acid (IS2)	17.5	310.1 -> 279.1
3,5-diMeO, 4-OH cinnamic acid	17.7	368.1 -> 338.1
flavanone	18.4	222.0 -> 194.0
diethyl stilbestrol	18.5	412.2 -> 383.2
6-methylflavone	19.1	236.1 -> 208.1
Resveratrol	20.1	444.2 -> 355.2
Phloretin	20.2	342.2 -> 327.2
Formononetin	20.6	340.2 -> 267.1
Epicatechin	20.9	650.2 -> 355.2
Naringenin	20.95	560.2 -> 545.2
Catechin	21.05	650.2 -> 355.2
Biochanin A	21.06	413.2 -> 398.2
Genistein-d4	21.38	475.1 -> 403.1
Hesperetin	21.4	575.1 -> 545.1
Genistein	21.41	471.2 -> 399.1
Daidzin	21.55	398.1 -> 383.1
Taxifolin	21.57	649.1 -> 368.1
Eriodictyol	21.59	560.2 -> 369.0
Kaempferol	22.25	559.2 -> 487.2
Chlorogenic acid	22.3	345.1 -> 255.1
Apigenin	22.7	471.1 -> 399.1
Quercitin	23.08	647.2 -> 575.2
Isorhamnetin	23.15	589.2 -> 559.2
Diosmetin	23.5	501.2 -> 471.2
Myricetin	23.5	647.2 -> 575.2
Luteolin	23.75	559.1 -> 399.1
Diosmetin	23.5	500.5 -> 471.0
Myricetin	23.5	734.5 -> 647.0
Luteolin	23.75	574.5 -> 559.0

The final temperature program is described in Table 4-6. The sample was injected at an initial oven temperature of 100ºCelcius and held for 1.5 minutes. The ramp rate was then raised at various increments until the oven reached 262ºCelcius, where it was held for 4minutes, and then raised again to reach 290ºCelcius and held for 3 minutes to achieve adequate separation of the compounds.

Table 4-6 Final temperature program utilised to achieve adequate separation of compounds

Ramp rate (ºCelcius/min)	Next Temp (ºCelcius)	Hold (min)	Actual Run Time (min)
	100	1.5	1.5
10	140	0	5.5
15	150	0	6.17
2	160	0	11.17
15	215	0	14.84
20	262	4	21.19
10	290	3	26.99

4.7.2 Precursor ion identification

After separation by GC, the molecules are eluted into the MS. The MS first operates by selecting precursor ions of a specific mass, to separate the ions from the chemical background. To identify the most appropriate precursor ion of the compounds, the mass analyser was set in an all-scan mode without any collision energy. For each of the compounds, the precursor ion was identified in the spectrum. An example of this scan output is in Figure 4-6, for Cinnamic Acid at 8.6m.

From the obtained spectrum for each compound, four ions were selected to be used in further collision experiments to identify appropriate product ions for each precursor ion. The criteria for selecting ions were to be abundant and of high mass. For example, the ions selected for cinnamic acid were *m/z* 220, 205,161 and 145 (Figure 4-6).

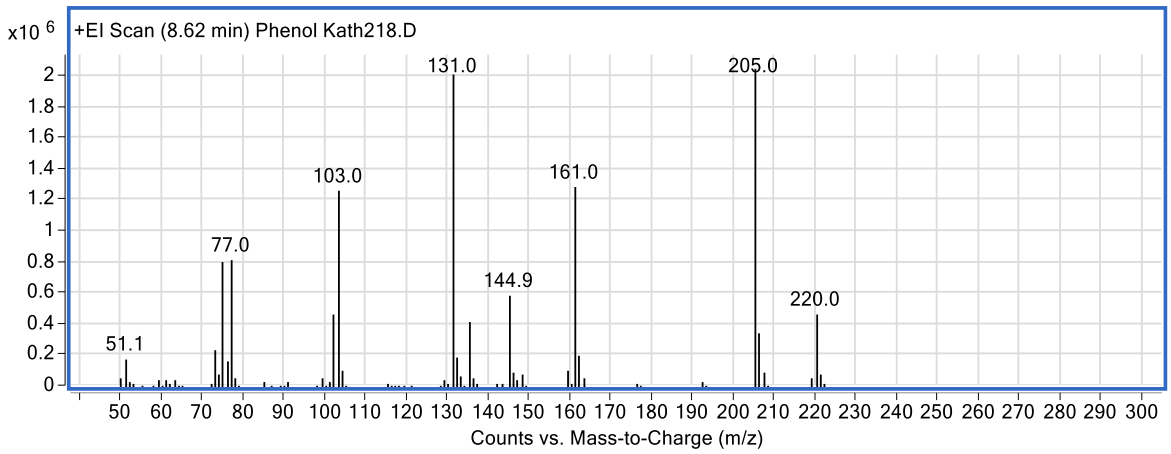


Figure 4-6 Mass spectrum for Cinnamic Acid at 8.6m

4.7.3 Collision energy experiments

The precursor ions are then induced to further dissociate through collision energy experiments (Figure 4-7), with all compounds and their four respective ions being analysed in separate experiments with four different collision energies (2, 6, 15 or 30 eV). This experiment is used to evaluate the best product ion generated from each identified precursor ion for each compound. The most appropriate collision energy generates a fragment that is smaller than the precursor ion, but still of reasonably high mass and abundance. This result is called a 'transition', which is written as precursor ion mass-> product ion mass, with each compound providing a unique ion spectrum.

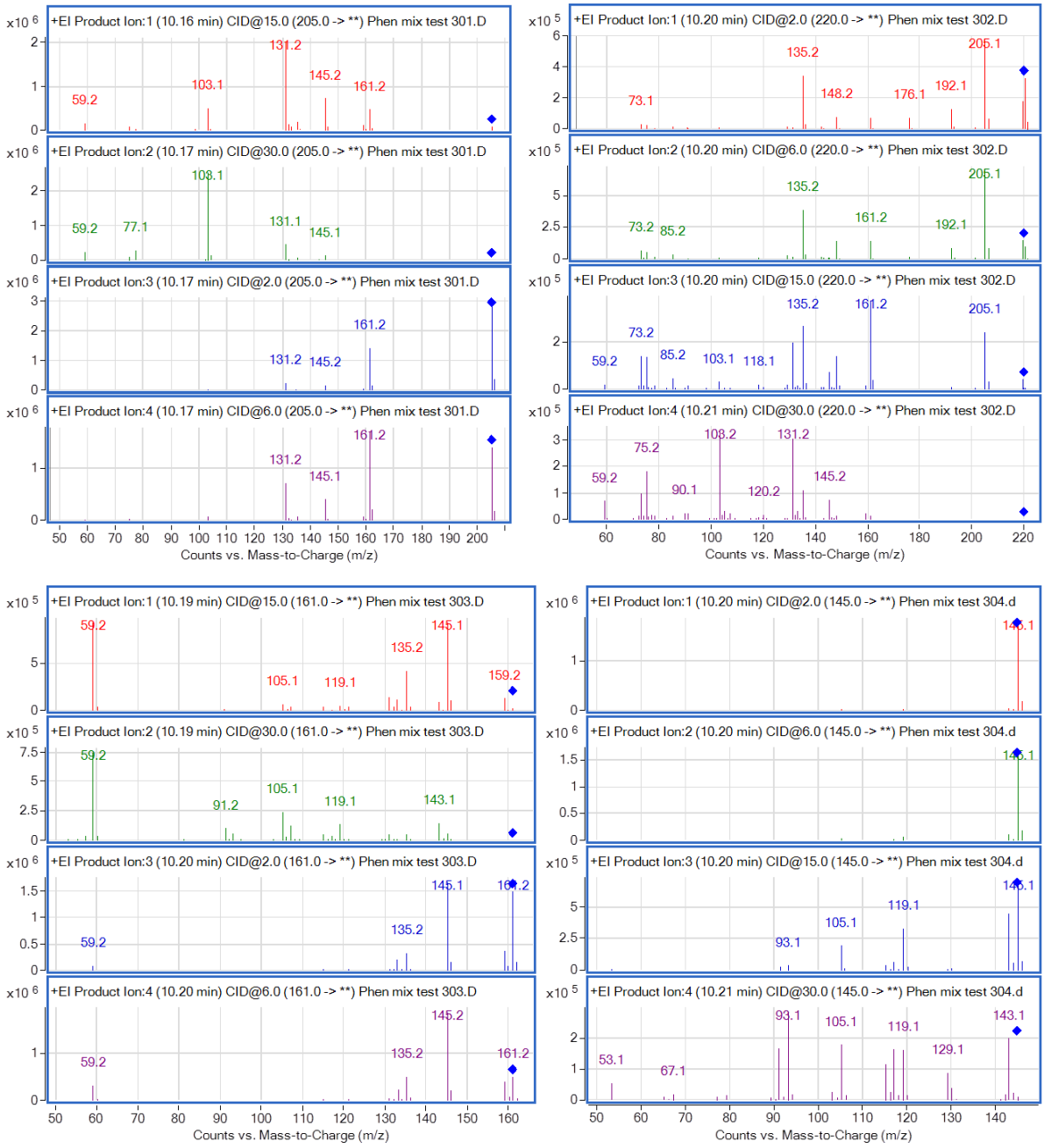


Figure 4-7 Collision energy experiment output for Cinnamic acid; the ions in each collision experiment are 220, 205, 161, 145 at CE 2, 6, 15 and 30 eV.

After the initial collision energy experiments, a multiple reaction monitoring (MRM) experiment is conducted to assess the most suitable collision energy from nine different collision energies (2.5, 5, 7.5, 10, 15, 20, 25, 30, 40 eV) for each selected transition. The collision energy which produces the greatest signal detected is selected as the most appropriate. An example of Cinnamic Acid is shown in Table 4-7.

The most appropriate transition and corresponding collision energy to be utilised is selected for each compound and is recorded for application in future analysis. The most appropriate transition for each compound analysed was selected and is displayed in table 4-5.

Table 4-7 MRM experiment output for all Cinnamic Acid transitions

Transition (precursor ion -> product ion)	**Collision Energy**	**Signal**
205.0 -> 161.2	4	1300000
205.0 -> 145.1	14	5200000
205.0 -> 131.1	14	13000000
205.0 -> 103.1	32	11000000
220.0 -> 205.1	4	3000000
220.0 -> 192.1	0	450000
220.0 -> 161.2	14	1300000
220.0 -> 135.2	4	1300000
220.0 -> 103.2	36	1100000
220.0 -> 75.2	24	380000
220.0 -> 73.2	24	90000
161.0 -> 145.1	4	7600000
161.0 -> 135.2	14	200000
161.0 -> 159.2	4	1300000
161.0 -> 105.1	32	710000
161.0 -> 59.2	16	3700000
145.1 -> 119.1	18	1400000
145.1 -> 129.1	36	340000
145.1 -> 105.1	24	720000
145.1 -> 93.1	32	700000

Finally, calibration curves were analysed using a blank matrix sample to determine whether the compounds added to the analysis were present. Calibrations curves for all compounds were obtained, with most producing good linearity, and some showing mixed linearity.

4.8 Discussion

The chapter initially describes the potential of measuring flavonoids and phenolic acids in biological samples as biomarkers of flavonoid intake, and the characteristics of GC-MS as a method to measure flavonoid intake are explained and evaluated. Additionally, this chapter describes the development of a GC/MS technique for the detection of 115 phenolic acids and flavonoids in biological samples via the construction of an in-house database of spectra and optimisation of the GC and MS methods. This method has been applied to measure some specific phenolic acids in human plasma to confirm the consumption and uptake of potential bioactive compounds in an anthocyanin-rich cherry juice, described in chapter 6 of this thesis, and the details of its application are reported in chapter 6.

A GC-MS method for the determination of phenolic acids and flavonoids in biological samples provides a sensitive and specific technique (24) and is a superior method for the separation of compounds (24). A sensitive and specific tool for the measurement of phenolic acids and flavonoids in biological samples is important, as the compounds are structurally similar and are present at micromolar to sub-micromolar levels (24).

The ability of a GC-MS method to measure biomarkers to provide an accurate indication of total dietary flavonoid exposure or quantify the intake of specific subclasses requires further investigation. However, the potential of biomarkers to indicate dietary intake of specific foods, such as tea (26), is more frequently studied, and more thoroughly understood.

Given the limitations that surround dietary measurement of flavonoids, it is important to develop methods to investigate the impact of flavonoids on health through measurement of intact flavonoids or related metabolites (to be used in conjunction or as stand-alone measures). The development of dietary biomarkers as an objective measure of food intake or exposure to bioactive molecules (4) is of significance for dietary studies, and in particular are useful when little data exists surrounding bioactive molecules and their metabolism (4).

Traditionally, a single biomarker is selected to measure dietary exposure to a food component. For example, vitamin C is often used as a measure of fruit consumption (4). However, early metabolomics studies have indicated that the use of a combination of several biomarkers may

improve the specificity of dietary exposure measurements (4), and could be applied when investigating the intake of single compounds, foods or potentially even dietary patterns.

4.8.1 Conclusion

The limited knowledge regarding which biomarker(s) are indicative of total dietary flavonoid intake, or which biomarkers should be measured to provide a relevant indication of the bioactive components of a particular food, a comprehensive method that can quickly and reliably measure multiple biomarkers of intake would be valuable in human trials. In this instance, the developed method has the potential to measure up to 115 possible biomarkers, which has a large applicability for exploratory ‘metabolomics’ related studies.

4.9 References

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CHAPTER 5: FOOD-BASED ANTHOCYANIN INTAKE AND COGNITION IN HUMAN INTERVENTION TRIALS: A SYSTEMATIC REVIEW

Pre-clinical evidence suggests anthocyanins, a subclass of dietary flavonoids, that provide the purple and red pigmentation in plant-based foods, may beneficially impact cognitive outcomes. A systematic review was conducted to summarise and synthesise the published literature on food-based anthocyanin consumption and cognitive outcomes in human intervention trials. Four studies were included in this review, comprising acute trials (n=2) and longer-term (n=2) interventions which assessed multiple cognitive outcomes in children, adults and older adults with cognitive impairment. Three out of 4 studies reported improvements in either a single, or multiple cognitive outcomes after anthocyanin-rich food consumption. Due to methodological limitations and the large clinical and methodological diversity of the studies, pooling of data was not feasible. The impact of food-based anthocyanin consumption on both acute and long-term cognition appears promising. However, adequately powered studies that include sensitive cognitive tasks are needed to confirm these findings and allow translation of research into dietary messages.

This manuscript has been updated and submitted for publication (Appendix G) :

> Kent, K, Charlton, K, Netzel, M, Fanning K 2015. The impact of food-based anthocyanin consumption on cognition in human intervention trials: a systematic review. (Journal of human nutrition and dietetics)

KK designed and conducted the review, and prepared the manuscript. KC, MN and KF provided critical revisions of the manuscript.

5

5.1 Introduction

The rapidly aging population has led to a major projected increase in the prevalence of neuro-degenerative diseases such as dementia (1), and resulted in intense research interest regarding the role of diet in neuro-protection. Dietary approaches are highly regarded as safe and effective methods for the prevention of cognitive disorders and the maintenance of cognitive function. The consumption of dietary flavonoids has been shown to both enhance cognitive function (2) and prevent age-related cognitive decline in older adults (3). The neuroprotective effects of flavonoids have been well-documented (4-8), but most of the research has focused on animal models, limiting translation to humans who utilise more complex cognitive domains, such as executive functioning. Due to their strong antioxidant potential (9), it has been hypothesised that flavonoids limit neurodegeneration by reducing neuroinflammation and protecting cellular architecture in the brain (10). Flavonoids may also contribute to improved cognitive function by increasing the number and strength of neuronal signals (11), through increased brain blood flow (7) and an ability to initiate neurogenesis (5) in areas of the brain that are associated with learning and memory. A large proportion of this research has specifically highlighted the benefits of anthocyanin-rich foods (12-15).

Anthocyanins are a subclass of dietary flavonoids (different from proanthocyanidins, which are polymers of flavanols), which provide the purple, red and blue pigmentation in plant based foods (16). Typical food sources of anthocyanins are fruits such as apples, grapes, plums; berries including strawberries, blueberries and blackberries; vegetables such as red cabbage, red onion, eggplant, and beverages including red wine and grape juice (17). Approximately 640 individual anthocyanins have been identified to date(18), however anthocyanins naturally occur most ubiquitously in six common agylcones (pelargonidin, cyanidin, delphinidin, peonidin, petunidin, malvidin) in various glycosylated and acylated forms (18). Anthocyanins are unique among flavonoids due to their ability to reversibly change their structures in response to changing pH values, from the stable red to orange coloured flavylium cation at pH <2 to the colourless and less stable carbinol and chalcone forms at higher or neutral pH (18-20). This unique feature may impact their stability and binding ability (e.g. to enzymes) throughout the gut which has a dramatically changing pH environment (“acid” stomach to “neutral” intestine).

The metabolism, absorption and bioavailability of anthocyanins is unique to other flavonoids and has been well documented (21). Briefly, food based anthocyanins are consumed and are either quickly absorbed as intact molecules via the stomach, or after extensive metabolism in the small and large intestine or the colon, to enter the circulation as metabolites such as phenolic acids (21). In previous animal and human studies, only very low amounts of intact (non-metabolised) anthocyanin glycosides could be detected in plasma and urine after ingestion of pure anthocyanin compounds and/or anthocyanin-rich food and beverages (15 animal and 17 human bioavailability studies were comprehensively reviewed by Pojer et al., 2013(19)). However, recent human studies have clearly demonstrated that dietary anthocyanins undergo intensive metabolism after consumption and that those metabolites are the main transport forms in vivo (22-25). For example thirty-two anthocyanin-derived metabolites could be detected in urine, serum and faeces of eight healthy volunteers after the consumption of 500 mg of ^{13}C-labelled cyanidin-3-glucoside(23). In another study published by Ludwig et al. (22)17 metabolites were quantified in urine collected from nine volunteers after the consumption of 300 g blended raspberries(22). Previously, bioavailability of anthocyanins was considered to be very low (urinary recoveries <2% of intake in most studies) but is now estimated as being at least 15% if detectable in vivo metabolites are also taken into account(22).

The main biological activity of anthocyanins in vivo was thought to be their antioxidant capacity and protection against lipid peroxidation (26). However, recent studies have identified more complex molecular effects of anthocyanins on signalling pathways in cells that may play a significant role in their protection against chronic diseases (19, 26, 27). Modulation of neuronal functions by anthocyanin-rich food has been reported in several animal studies (cited and reviewed by Pojer et al., 2013(19)). It is suggested that these neuroprotective effects (e.g. improved memory, learning, cognitive and motor functions) by anthocyanins (and/or their in vivo metabolites) are mediated by their ability to cross the blood-brain barrier and act to inhibit/supress interleukin-1b (IL-1b), tumor necrosis factor-alpha (TNF-α), and nuclear factor kappa B (NF-κB) (19). Furthermore, modulatory effects of anthocyanins on important structural and synaptic plasticity markers have also been reported.

A body of evidence about the benefits of anthocyanin consumption in human health, including protective effects against age-related neurodegeneration and cognitive decline, has been accumulating over the past few years (16, 19). Animal studies have provided evidence that

anthocyanin-rich food consumption can improve several cognitive functions, including long-term memory (28), spatial-working memory (29), object-recognition memory (30). In addition, improvements in motor performance (i.e. balance and coordination) have been documented after an anthocyanin-rich blueberry supplemented diet in an animal model (14). Intact (non-metabolised) anthocyanins were been detected in the brains of rats as soon as ten minutes after their consumption, and these concentrations were found to be correlated with improvements in learning and memory tasks (13). Of particular interest is that supplementation of the diet with anthocyanin-rich fruits, such as blueberries and strawberries, has been shown to not only maintain, but also reverse, age-related cognitive decline in animal models (11, 31, 32).

A large epidemiological study of 16,010 older women enrolled in the Nurses Health Study reported significant associations between anthocyanin-rich blueberry and strawberry consumption and maintenance of cognitive function (a composite score of 6 cognitive tests, including various measures of memory) over 4 years (33). It was reported that those who consumed the highest amount of berries had delayed cognitive aging by up to 2.5 years, compared to those who ate the least (33). Despite their known health benefits, anthocyanins are often overlooked in human intervention trials that investigate the impact of overall flavonoid intake on cognitive outcomes (34). In a 2009 systematic review of human intervention trials assessing the impact of flavonoids on cognitive outcomes (34), no studies related to anthocyanins were included.

There has been an increasing interest in other areas of anthocyanin research, with a 15-fold rise in the number of published papers between 1990 and 2013 having the word “anthocyanin” in their title and abstract (35) Important advances in an increased understanding of the bioavailability and metabolism of anthocyanins (e.g. biotransformation by the gut microbiota), coupled with a greater focus on the role of plant foods in brain health (e.g. neuroprotective activities), warrant a targeted review to summarise the evidence to date of human trials on anthocyanins and cognitive function.

5.2 Objective

The aim of this systematic review is to summarise and synthesise the published literature on food-based anthocyanin consumption and cognitive outcomes in human trials. The objectives of the review are to:

a) Examine the effects of food-based anthocyanin consumption on cognition and;
b) Highlight the gaps in the literature and address the implications for future studies

5.3 Methods

A systematic review of the literature was performed using the following strategy.

5.3.1 Types of studies

Studies were considered for inclusion in this review if they:

- had an experimental design (randomised or non-randomised controlled trials; cross-over trials)
- examined humans (any age, gender);
- administered an anthocyanin-containing food or beverage (amount of anthocyanin objectively measured);
- measured change in cognitive function using a single, or multiple valid and reliable tool/s;
- included a control group;
- had full text available.

Only studies written in the English language were considered due to a lack of translational resources, but studies from any country were eligible if they met the rest of the inclusion criteria.

5.3.2 Search terms

The following search terms were developed:

- Anthocyan* and/or
- Flavonoid* and
- Cognit* and/or
- Memory

The * indicates a truncation, to ensure all variations of the search terms were included.

5.3.3 Databases

A three stage search was conducted. Firstly, a search of the following databases (abstract, title and keywords) was performed using the search terms using the Boolean terms AND/OR as appropriate (Table 5-1):

- Scopus (1960 – Sept 2015)
- Medline (1960 – Sept 2015)
- CINAHL (1960 – Sept 2015)
- Psychology and Behavioural Sciences Collection (1960 – Sept 2015)
- PsychInfo (1860 – Sept 2015)
- Web of Science (1965- Sept 2015)

Note: the differing search years relate to the database availability

The second stage consisted of a hand search of the reference list of identified studies. A third search consisted of an internet search to identify any studies published outside the realms of the available databases. This website searched was google scholar (www scholar.google.com.au) using the aforementioned key words (1960-Septermber 2015). The records were managed in EndNote X4 software.

5.4 Results

The search (Table 5-1) returned 1,684 articles, and one other potential study was identified through hand-sorting of reference lists. After duplicates were removed and the titles screened, 32 potential studies were identified. After screening abstracts a further 25 were excluded (Table 5-2) and after reading the full text articles of 7 studies, a further three were excluded (Table 5-2) according to the inclusion/exclusion criteria, leaving four final papers in the review (Figure 5-1).

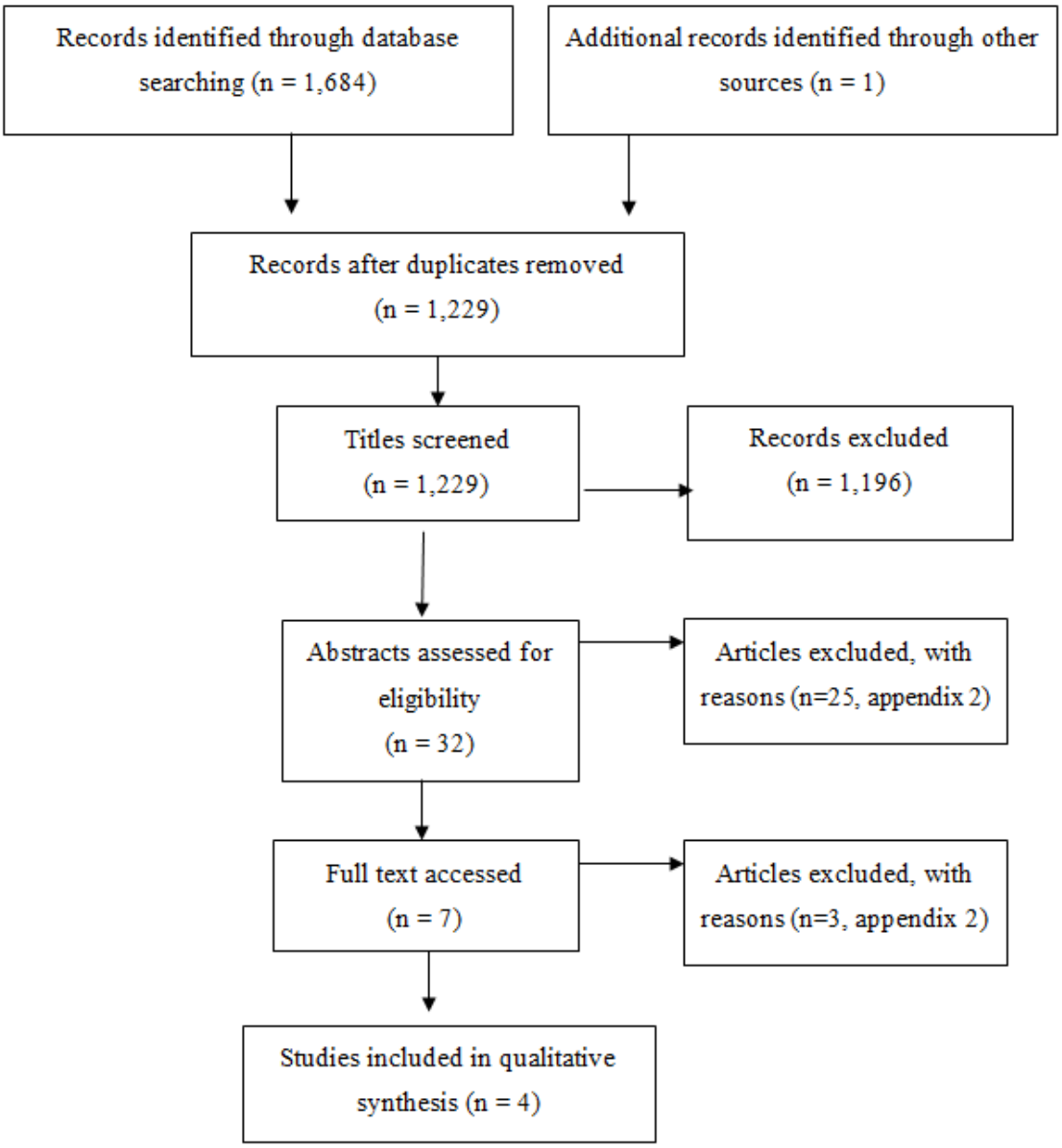

Figure 5-1 PRISMA flowchart for the search and study selection process of included and excluded studies

Table 5-1 Search strategy results

		Terms	# Results
PsychInfo	1	anthocyan*.mp. [mp=title, abstract, heading word, table of contents, key concepts, original title, tests & measures]	38
	2	flavonoid*.mp. [mp=title, abstract, heading word, table of contents, key concepts, original title, tests & measures]	285
	3	memory.mp. [mp=title, abstract, heading word, table of contents, key concepts, original title, tests & measures]	182671
	4	cognit*.mp. [mp=title, abstract, heading word, table of contents, key concepts, original title, tests & measures]	445379
	5	1 or 2	315
	6	3 or 4	556903
	7	5 and 6	87
Medline, CINAHL, Psychology and Behavioral Sciences Collection	S3	(memory OR cognit*) AND (S1 AND S2)	639
	S2	memory OR cognit*	663,419
	S1	anthocyan* OR flavonoid*	54,144
Web of science	3	#2 AND #1 Indexes=SCI-EXPANDED, SSCI Timespan=All years	61
	2	(TI=(cognit*) OR TI=(memory)) *AND* **LANGUAGE:** (English) Indexes=SCI-EXPANDED, SSCI, A&HCI, CPCI-S, CPCI-SSH, CCR-EXPANDED, IC Timespan=All years	20,397

	1	(TI=(anthocyan*) OR TI=(flavonoid*)) *AND* **LANGUAGE:** (English) Indexes=SCI-EXPANDED, SSCI, A&HCI, CPCI-S, CPCI-SSH, CCR-EXPANDED, IC Timespan=All years	298,592
Scopus		((TITLE-ABS-EY (anthocyan*) OR TITLE-ABS-KEY (flavonoid*))) AND ((TITLE-ABS-KEY (cognit*) OR TITLE-ABS-KEY (memory)))	897

Table 5-2 Excluded studies

		Study reference	Reason
Excluded after abstract reviewed	1	(36)	No measure of cognition
	2	(37)	No measure of anthocyanin in apples
	3	(38)	Animal study
	4	(39)	Not primary research
	5	(40)	Not primary research
	6	(41)	Animal study
	7	(42)	Conference abstract
	8	(43)	Animal study and non-food based
	9	(44)	Conference abstract and study design
	10	(45)	Review, not anthocyanin specific
	11	(46)	Conference abstract
	12	(47)	Editorial
	13	(48)	Review of blueberries, not focussed on cognition
	14	(49)	Full text cannot be found
	15	(50)	Animal study
	16	(51)	Pomegranate extract in pill form
	17	(52)	Narrative review focussed on animal studies (small section on Krikorian papers identified)
	18	(53)	Animal study
	19	(54)	Review of grape products, not focussed on cognition
	20	(55)	Review of animal studies
	21	(56)	Animal study
	22	(57)	Intervention not flavonoid related
	23	(58)	Review, cognitive outcomes not addresses
	24	(59)	Review, not food vehicle
	25	(60)	Animal study, black soy contains no anthocyanin
Excluded after full text retrieved	26	(61)	No measure of anthocyanin in juice
	27	(62)	No measure of anthocyanin in juice
	28	(63)	No measure of anthocyanin in juice

5.4.1 Description of studies

Four studies were deemed eligible for the review (64-67). For this review, statistical pooling of the data was not possible due to the large clinical and methodological diversity (variability in the participants, interventions, study design and outcomes studied) leading to a lack of homogeneity of the studies, therefore the findings are presented in a narrative form. The characteristics of each study are described in Table 5-3 and relevant information summarised in Table 5-3. The publication dates ranged from 2010-2015. Of the four included studies, two were randomised, double blind, placebo controlled studies (64, 65) that assessed chronic daily consumption of anthocyanin-rich juices on cognitive outcomes, and one was a randomised (67) and the other a non-randomised (66) placebo-controlled crossover trial that assessed the acute impact of anthocyanin rich juices on cognitive outcomes. Two studies investigated older adults with mild-cognitive impairment (MCI) (64, 65), one study was conducted in young, healthy adults (67) while another was undertaken in children aged 8-10y (66). The studies were undertaken in New Zealand (67), the USA (64, 65) and the UK (66).

5.4.2 Description of the interventions

In all four studies, the vehicle for anthocyanin administration was a beverage. Two studies utilised a pre-formulated juice (64, 65) and placebo control, one study (67) utilised a juice processed from fresh fruit (150g fruit yielded ~140ml juice) and a powdered fruit extract made up with water, and one study (66) blended fresh fruit into a smoothie. Two studies based the beverage serving size on the body weight of each participant (64, 65) providing between 6ml – 9ml of juice/kg body weight of juice (providing between 428mg-598mg anthocyanin/day divided over three servings). One study (67) altered the serving sizes of the juice to consistently deliver 525±5 mg of polyphenols per 60 kg of bodyweight per participant. One study (68) administered a standard serving size to each participant (143mg anthocyanin/serve), regardless of body size.

Table 5-3 Included studies

Author	Participants	Design	Intervention	Dose	Cognitive tests	Results
Krikorian et al., 2012 (64)	Krikorian et al., 2012 (64)	21 older adults, 68y+, mild cognitive impairment (clinical dementia rating scale)	Randomised double-blind placebo controlled trial	16 weeks, daily consumption of concord grape juice (n=10) vs placebo (n=11) (colour, taste, calorie & sugar matched)	Anthocyanins: 425mg/L (spectrophotometry). Serving size calculated to be between 6 & 9ml/kg body weight over 3 servings/day	Baseline & 16 weeks: California Verbal Learning Test II (verbal learning & memory), Geriatric depression scale (mood), Sub-group (4 per group) fMRI brain imaging
Krikorian et al., 2010 (65)	Krikorian et al., 2010 (65)	9 older adults (5 men, mean age 76.2±5.2) mild cognitive impairment (clinical dementia rating)	Randomised, double blind, placebo controlled trial	12 weeks, daily consumption of blueberry juice (n=9) vs placebo (n=7) (not matched, control group from another study (62)).	Anthocyanins: 877mg cyaninidin 3-glucoside equiv/L (HPLC). Serving size between 6 & 9ml/kg body weight over 3 servings/day	Baseline & 12 weeks: California Verbal Learning Test (verbal learning & memory), Verbal paired associate learning test (memory)
Watson et al., 2015 (67)	36 young adults (mean age 24.8y ± 3.9)	Acute randomised double-blind placebo controlled crossover study	3 juices on different occasions: placebo juice, blackcurrant extract or blackcurrant juice	Placebo juice: 0mg/kg anthocyanin; Blackcurrant extract: 7.7mg/kg anthocyanin; Blackcurrant fruit	7 repetitions over 70m post intervention: digital vigilance (attention), Stroop (attention, inhibition), rapid visual information processing (RVIP, attention &	Sig. improvement in RVIP accuracy after supplementation with anthocyanin-enriched blackcurrant extract vs control (p= 0.011). Sig. improvement in reaction

				juice: 8.0mg/kg anthocyanin. Serving size 10ml/kg body weight	working memory), Bond-Lader visual analogue (mood)	time on some repetitions (1, 4, 7) on digit vigilance task vs baseline for the cold-pressed blackcurrant fruit juice (overall effect p=0.044). No sig. effect on mood
Whyte et al., 2015 (66)	16 children (10 male, mean age 9.17y ± 0.6)	Acute cross-over placebo controlled trial	Intervention: 200g fresh blueberries, 100mL milk & 8g sucrose. Control (sugar & vit C matched): 0.02g vit C powder, 8.22g sucrose, 9.76g glucose & 9.94g fructose & 100mL milk	Intervention: 143 mg anthocyanins (method not described)	Baseline and 2h post intervention: Go-No Go (response inhibition), Stroop (verbal reaction & interference), RAVLT(verbal learning & memory), Object Location Task (spatial memory & discrimination), & a Visual N-back (visual attention & short term memory)	Sig. improvement in delayed recall of RAVLT (word learning & retention) for intervention group (p=0.038). No sig. improvement for other tasks

5.4.3 Outcome assessment

Cognitive outcomes were measured using valid tools in all studies. Two studies measured acute change in cognition (66, 67) while the other two studies measured longer term change in cognition over 12 to 16 weeks (64, 65).

5.4.4 Longer term anthocyanin supplementation and cognition

The longer-term studies both evaluated verbal learning and memory in older adults with MCI (64, 65) using the California Verbal Learning Test, one study measured change in explicit episodic memory using Paired Associate Learning Test (65) and one study assessed change in mood using the Geriatric Depression Scale (64). One study (64) measured cognition objectively, using fMRI brain imaging, which indicates stimulation and activity in specific regions of the brain.

Krikorian et al., (64) reported that after 16 weeks supplementation with concord grape juice, there was no between-group difference for performance on the CVLT learning task, but there was a reduction in interference on the CVLT memory task compared to baseline ($p=0.04$). There was a NS effect on mood. Analysis of the fMRI brain analysis indicated that there was greater activation in the anterior and posterior regions of the brain for the intervention group only (64). In another study, the same research team of Krikorian et al., (65) reported significant improvements on the verbal paired associate learning task ($p=0.009$) after 12 weeks of supplementation with blueberry juice. However, both of these studies had small sample sizes of only $n = 10$ in the Concord grape juice intervention arm (64) and $n = 9$ in the blueberry juice intervention arm (65).

5.4.5 Acute anthocyanin supplementation and cognition

The two acute feeding studies evaluated change in acute cognition using the Digital Vigilance test (a measure of attention) (67), the rapid visual information processing test (attention and working memory) (67), the Go-NoGo test (response inhibition) (66), Rey Auditory Verbal Learning Task (RAVLT) (verbal learning, memory) (66), an objective location task (spatial memory) (66) and a visual-n back test (attention and short term memory) (66). Both studies used the Stroop test to measure attention and inhibition (66, 67).

Watson et al., (67) compared three conditions (placebo vs powdered blackcurrant extract with 7.7mg anthocyanins/kg vs blackcurrant juice with 8mg/kg, based on body weight) on acute cognition in 36 young adults by administering tasks seven consecutive times during a period of 65 minutes after consumption of the beverages. The authors reported significant improvements in accuracy as measured by the Rapid Visual Information Processing test ($p=0.011$) when participants consumed the blackcurrant extract vs placebo, and improvements in reaction time for some repetitions (1, 4 and 7) on the digital vigilance task compared to baseline tests ($p=0.044$) for blackcurrant juice or extract vs placebo. No change in mood or other tasks was recorded.

Whyte et al., (66) assessed acute change in cognition after consumption of 200g fresh blueberries (143 mg anthocyanins) provided in a milk-based smoothie in 16 children. There was a significant improvement in the delayed recall portion of the RAVLT task after consumption of the blueberry smoothie ($p=0.038$), but no improvements seen for any other task.

5.5 Discussion

The aim of this systematic review was to assess the effects of anthocyanins consumed from food, not supplements, on domains of cognitive function, as well as highlight gaps in the literature and address the applicability of the available evidence for development of future study designs. Despite over a decade of pre-clinical evidence supporting the association of anthocyanin intake on cognitive outcomes (69), there remains a lack of experimental studies conducted in humans. The four studies included in the review reported positive benefits of anthocyanin consumption on some aspects of cognition; however the interpretation of the studies is limited by small sample sizes and inconsistent methodologies.

There remains a lack of consensus regarding which cognitive domain is impacted by flavonoid (including anthocyanin) intake. This limits choice of best instruments and methods to employ in studies that investigate potential change in cognitive function (34). The four identified studies used a wide range of tools to address multiple cognitive domains. This inconsistency limits comparability between studies and prevents the study findings from being synthesized.

This limitation has been recognised, as pointed out in a recent methodological review paper (70). The review concluded that future research should be guided by positive findings from previous similar research and that it may be best to continue to include a battery of sensitive and specific tools that address multiple cognitive domains, until there is better understanding of specific brain functions that are influenced by these bioactive components in foods.

The length of the intervention needed to demonstrate outcomes in both acute and chronic trials is also poorly understood. The results of the studies by Krikorian and colleagues (64, 65) indicate that an intervention of 12 weeks is long enough for cognitive improvements to be observed, and that extending study to 16 weeks may not improve the strength of the association. The timing related to the acute impact of food-based anthocyanins on cognition is hampered by the limited knowledge related to their metabolism and the exact intrinsic effects of anthocyanin-rich foods. Understanding to date of anthocyanin metabolism (71) suggests that a small proportion of anthocyanins are absorbed within 1-2h of consumption and that the remainder pass through to the colon (at 5-6h post consumption), where they are extensively metabolised by colonic microflora into metabolites with many potential bioactivities. It is not yet clear whether the intact anthocyanins or their metabolites, or both, are responsible for the beneficial effects of an anthocyanin-rich diet (72). The two acute studies reviewed in this paper did not extend their trial times to encompass the secondary uptake of anthocyanin-related metabolites in the colon, and thus may have missed an important later interaction that these compounds have on cognition.

Another outcome from our systematic literature review is that biomarker data for anthocyanin uptake is largely unavailable. Only one study (67) conducted an objective analysis of biological specimens to determine the uptake and metabolism of the anthocyanin present in the intervention foods. This fundamental data needed to confirm the bioavailability of anthocyanins from different foods and to determine which of the bioactive components are specifically associated with the observed health benefits. Current information suggests that the level of absorption of flavonoids, and especially anthocyanins, is very low (73) which makes them difficult to detect in plasma and urine samples. Identification of the full complement of phenolic acid metabolites produced from the breakdown of anthocyanins has not yet been made.

Different age groups may respond differently to anthocyanin intake. The studies included in this review investigated groups ranging in age from children (8-10y) to older adults (>68y) with mild-cognitive impairment. Disease states that affect brain function, including vascular and non-vascular dementia, as well as Alzheimer's disease may also result in different measureable outcomes in these groups (74), as shown by the limited impact of antioxidant clinical trials in this group(74). Therefore, it is important to clearly define patient groups and the differences in the effect between children, and young and older adult's needs to be investigated.

The provision of anthocyanins through a juice/beverage vehicle was not well explained in all of the studies. It can be speculated that the provision of a pre-formulated juice is an ideal way to control the variation of anthocyanins in the fresh-fruit equivalent, which can be heavily influenced by growth conditions and can differ between seasons. Provision of a juice also reduces the impact of fruit seasonality on intervention timing. However, the impact of food processing can substantially alter the anthocyanin content of foods, especially with regard to heating or cooking. For example heating of dark anthocyanin-rich blood plums during jam making resulted in losses of up to 70% of their total anthocyanin content (75). Additionally, some studies administered relatively large doses of anthocyanin-rich beverages (up to over 600ml), presumably to maximise the potential of the bioactive anthocyanins. The division of the anthocyanin-rich food over 3 servings per day in the longer-term trials (64, 65) may have diminished their bioactivity. The provision of smaller doses may not have provided a sufficient concentration of anthocyanin to exert a physiological effect, compared to an increased bioactivity if a larger serve is consumed at once. However the dose-response mechanisms for the impact of anthocyanin-rich food on cognition have not been well investigated. There is a trade-off in clinical trial methodology between reflecting usual intake of anthocyanin-rich foods, as spread throughout the day, and ensuring a sufficiently high dose is provided (in a single serving) to observe outcomes. Future trials are needed to further elucidate the dose-dependent responses associated with consumption of anthocyanin-rich foods.

Background diet was considered by only one of the intervention studies(76), whereby recommendations were provided on which foods to avoid the evening prior to testing day. Diet was controlled on the day of the intervention in the longer acute trials (76, 77), but was not controlled in the longer-term trials. Controlling the background diet, or at least monitoring it,

is an important consideration in flavonoid trials (78) in order to isolate the effect of the intervention. Provision of low flavonoid and/or washout diets before and during trials may be an effective way to reduce the impact of habitual diet on intervention outcomes, although monitoring habitual diet throughout the period of longer term interventions may also be useful to identify potential dietary confounders (78). However, the ideal protocol for standardising with the background diet, including the length of run-in periods in flavonoid trials has not been investigated and identified, although a minimum of 3 days has been suggested (78).

Berries, particularly blueberries and blackberries, have received the most interest in studies investigating the health impacts of anthocyanin-rich foods. Many other anthocyanin-rich sources have been overlooked, including vegetables such as red onions and cabbage. The potential for these foods to influence cognition needs to be confirmed, as the large dependence in research on berries limits the translation of the outcomes to nutritional advice for the population. The consumption of large amounts of berries is not always feasible, as berries are often expensive, seasonal (not available year-round) and can spoil quickly. The use of freeze-dried fruits to overcome these barriers may be a possibility, providing that the anthocyanin content of the fresh fruit equivalent is not spoiled. Freeze-dried fruit may be a nutritionally more preferable way in which to consume anthocyanins, compared to encapsulated anthocyanins or anthocyanins added to other processed foods. This is because anthocyanin pigments present within natural foods are unlikely to be working independently, as plants typically contain a complex mixture of phytochemicals that have synergistic bioactivities (79). Some studies have found that anthocyanin bioactivity may be increased when they are consumed within a mixture of polyphenols, for example as they would naturally occur within a complex food matrix (80). This emphasises the importance of investigating whole food sources of anthocyanins, rather than isolated and encapsulated anthocyanins when studying health related outcomes.

The implication of study design (ie a cross-over design versus parallel groups) is important when considering flavonoid trials. Due to the high intra-individual variability in the metabolism of flavonoids (81), a cross-over design whereby each individual serves as their own control, is arguably more appropriate, especially in trials with small sample sizes.

The studies identified in this systematic review that were conducted by Krikorian et al.,(64, 65) have been criticised for using the placebo control results from one study, as the placebo control in the other(82). It may have been preferable to analyse this data three-arm study. Lamport et al., (82) has suggested that this method may reduce the significance of the results (loss of power from increased number of comparisons). Another limitation of these studies is the differences in mean age between the placebo and the control groups (5 years), which may be associated with the difference in cognitive performance.

Three studies were excluded on the basis of not having a measure of anthocyanin in the intervention juice (61-63). Hendrickson et al., 2008 (63) measured total phenolic content, but did not report anthocyanin content specifically. Despite previous research showing that both cranberry (83) and grape juice (64) contain anthocyanins, without an objective measure of their anthocyanin content the extent to which their bioactivities relate to the anthocyanin content cannot be speculated. These studies showed that in older adults (n=5), daily concord grape juice consumed (4-6ml/kg body weight divided over 3 servings per day) over 12 weeks was associated with significant improvements ($p=0.04$) in verbal learning and memory on the California Verbal Learning Test. In young adults (n=35), grape juice (10ml/kg body weight) had no impact on acute cognition (implicit memory as measured by a word fragment completion task) and cranberry juice consumption (~470ml consumed twice per day) had no significant impact on cognitive outcomes.

5.5.1 Conclusion

The impact of food-based anthocyanin consumption on both acute and long-term cognition appears promising. However, adequately powered studies that include sensitive cognitive tasks are needed to confirm these findings and allow translation of research into dietary messages.

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CHAPTER 6: FLAVONOID RICH CHERRY JUICE ACUTELY REDUCES BLOOD PRESSURE, BUT NOT COGNITION IN YOUNG ADULTS, OLDER ADULTS AND DEMENTIA PATIENTS: A PILOT CROSSOVER STUDY

This chapter describes a pilot cross-over study that was conducted to assess the acute effects on cognitive function, blood pressure and plasma biomarkers associated with consumption of a 300 ml flavonoid-rich fruit juice, provided either as a single quantity or as 3x100ml doses administered over 2h. Young adults (n=6), older adults (n=5), and older adults with dementia (n=5) received in random order either a single 300ml dose or 3x100ml doses of high-flavonoid cherry juice at 0, 1 and 2h. Blood pressure and plasma levels of phenolic metabolites were measured at 0, 2 and 6h. A battery of cognitive tests was administered at baseline and 6h. Regardless of dose, cherry juice did not impact acute cognitive function. The single 300ml dose of juice significantly reduced heart-rate (p=0.024) and diastolic blood pressure (p=0.016), with a trend for reduced systolic blood pressure (p=0.066) after two hours. The 3x100ml doses did not result in blood pressure reductions and no group effect was evident in either dose. Plasma phenolic metabolites increased at 2 and 6 hours, however changes were higher after the single 300ml dose. The methodology used in this acute pilot study contributes to study design for food-based trials of cognition. Flavonoids delivered in cherry juice did not acutely improve cognition; however a single 300ml quantity of juice reduced blood pressure in all groups similarly. The data suggests that dose-interval administration may be an important factor when administering the intervention, as a minimum threshold of uptake may need to be reached in order to induce acute bioactive effects.

This chapter has been published in two separate manuscripts, but will be presented here as a cohesive chapter.

Appendix A

> Caldwell* K, Charlton KE, Roodenrys S, Jenner A. 2015. Anthocyanin-rich cherry juice does not improve acute cognitive performance on RAVLT. Nutritional Neuroscience. DOI: 10.1179/1476830515Y.0000000005

Appendix E

Kent, K, Charlton, KE, Jenner, A, Roodenrys, S, 2015, Acute reduction in blood pressure following consumption of anthocyanin-rich cherry juice may be dose-interval dependant: a pilot cross-over study. Journal of Food Sciences and Nutrition. 67 (1), 47-52.

This data was also presented at national and international conferences:

Caldwell*, K., Charlton, K. E., Roodenrys, S. & Jenner, A. 2013. High anthocyanin cherry juice acutely impacts blood pressure but not cognition in young people, older people and dementia patients. Clinical Nutrition, 32 (Supplement 1), S122. Abstract presented at the 35th ESPEN Congress, 31 August 2013 - 03 September 2013, Leipzig, Germany

Caldwell*, K., Charlton, K. E. & Roodenrys, S. 2012. The acute impact of high anthocyanin cherry juice on cognition and blood pressure in young people, older people and dementia patients. Australasian Medical Journal, 5 (12), 723. Abstract presented at the Annual Scientific Meeting of the Nutrition Society of Australia, 27-30 November 2012, Wollongong, Australia

KK was responsible for the design of the study, organisation and leading of the study, data collection and analysis (including laboratory analysis) and preparation of the manuscript. KEC and SR were responsible for critical discussions of the study design and analysis, and critical revisions of the manuscript. AJ oversaw and advised the biomarker analysis and reviewed the manuscript.

6

6.1 Introduction

A growing body of evidence suggests that consumption of flavonoids, a group of polyphenols synthesised by plants is associated with enhanced cognitive function (1) and protection against neurodegenerative diseases (2). Dementia is a common mental and behavioural disorder in Australia and limited successful treatment options highlights the need for alternative practices to improve cognitive outcomes. Reviews of pre-clinical evidence have shown that flavonoids act to suppress neuroinflammation, improve cerebrovascular blood flow inducing angiogenesis and neurogenesis, as well as improve synaptic plasticity and scavenging neurotoxins and pro-inflammatory species in the brain (3, 4). Intense interest relates to class of flavonoids known as anthocyanins, that are concentrated in purple and red fruits (5). Anthocyanins have been shown to improve cognitive function (6-10), with various anthocyanin derivatives providing among the greatest antioxidant and free radical scavenging activity of all flavonoids (11). Sweet cherries are a rich source of anthocyanins and to a lesser degree contain flavan-3-ols, flavonols (12, 13) and phenolic acids (14) but have been overlooked in investigations of the cognitive effects of flavonoid and anthocyanin-rich foods.

6.1.1 Anthocyanins and acute cognition

While large scale epidemiological research has provided evidence that anthocyanin consumption is associated with improved cognition (15, 16) and slower cognitive decline (15) the ability of these bioactive compounds found in fruit and other foods to modulate acute cognition has yet to be fully examined. In the context of large-scale trials investigating the long-term cognitive benefits of flavonoid rich foods, focussed acute trials are also necessary to determine differences in acute and chronic effects.

Few well designed human trials have investigated the acute impact of flavonoid-rich food on cognition, and the development of appropriate study protocols is required. Notable gaps in existing knowledge relate to potential age differences associated with consumption of anthocyanins, variations in health outcomes related to dosage and timing of consumption, as well as identification of which cognitive domains are affected and the metabolic fate of flavonoid-rich foods.

Flavonoid consumption has been linked with cardiovascular benefits (17) and experimental studies indicate that the underlying biological mechanisms by which flavonoids regulate blood pressure include effects on vascular blood flow and vascular reactivity (18, 19). Regulation of blood pressure is an important clinical consideration for maintenance of cognition (20). Hypertension has been associated with poorer cognition in older adults (21, 22), which may relate to mechanisms that link flavonoid consumption to cognitive functioning. There is some evidence that suggests a weaker association between anthocyanin intake and cardiovascular outcomes in older adults (18) and additionally, the differences in acute responses between younger and older adults have not been adequately addressed.

There is no consensus regarding the quantity of flavonoids needed to induce bioactive effects and studies rarely consider a dose-timing analysis, with a few exceptions (23). The dose-dependent association on blood pressure reduction has not been supported (23) and is largely unexplored at the acute level. Studies often administer unrealistically large doses of flavonoid-containing foods in order to ensure a physiological result (8-10, 23), which limits translation into practical nutritional advice or generalisability to usual diets.

6.1.2 Measurement of flavonoid biomarkers in acute trials

Regarding biomarkers, only a few methods have been developed to measure the intake of flavonoids in biological samples, with little consistency in the literature. Flavonoids are quickly and extensively metabolised by the gut into phenolic acid metabolites (24), which may have different bioactivity to intact flavonoids. Plasma phenolic acids have been promoted as valid biomarkers of flavonoid intake (25, 26). Phenolic acids may reflect the absorption and metabolism of flavonoid rich foods and may be useful in understanding the metabolic fate of specific flavonoid rich foods (27).

The aim of this study was to develop and evaluate methodology to examine the acute impact of flavonoids from cherries on cognition and blood pressure; compare acute dose-timing effects of flavonoids from cherry juice on cognition and blood pressure in young people, older adults and older adults with mild to moderate dementia; and examine dose-timing effects relating to the absorption and metabolism of flavonoids from a feasible serving of cherries, over time and between groups, using Gas Chromatography/Mass Spectrometry.

6.2 Methods

Ethics approval was awarded by the University of Wollongong Human Research Ethics Committee. Participants were recruited through advertisements pursuing young healthy adults (18-35y) (n=6), older adults aged (55+y) (n=5) and older adults with dementia (n=5). Participants were screened for exclusion criteria of uncontrolled hypertension and dysphagia. Dementia diagnosis from a geriatric physician was required for inclusion in the dementia sub-group. Written informed consent was obtained and consent was provided by a carer when appropriate. Consenting participants were randomised to a treatment allocation and cognitive assessment order by computer-aided block randomisation by an independent statistician. Participants attended the study clinic on two occasions with a one-week washout period between visits.

On both occasions, participants fasted for 12 hours and were provided with a standardised breakfast with negligible flavonoids (wheat biscuits and milk). Cherry juice was administered either as a single 300ml at 0hrs or 3x100ml servings at 0, 1 and 2hrs. Cherry juice was produced from a single seasonal batch, underwent novel processing to minimally damage anthocyanin content and was frozen at ≤-0.20^{o}C. Oxygen Radial Absorbance Capacity of the cherry juice was 58.99 µmol Trolox Equivalent/g and the Anthocyanin content was determined to be 69mg/100ml using a validated HPLC (28) method. A standardised snack with negligible flavonoids (ham and cheese on white bread) was provided at 4 hrs.

6.2.1 Anthropometrics

Height and weight were measured and BMI calculated. Nutritional status was assessed using an interviewer-administered Mini Nutritional Assessment (MNA) (29). Resting blood pressure and heart rate were measured using an Omron HEM7200 Deluxe Automatic Blood pressure Monitor, while seated, in triplicate and averaged at baseline, 2 and 6 hours post-intervention.

6.2.2 Blood collection

Blood samples were collected in EDTA tubes at baseline, 2 and 6 hours post-intervention and centrifuged within 1 hour, with plasma extracted and stored at -0.80^{o}C until batch analysis.

Phenolic acid analysis was performed according to Loke et al, (25) on an Aligent-7000 Gas Chromatography/Mass Spectrometer-Triple Quad. Briefly, the collected blood was treated with 18 µl of indomethacin (5 mM). The sample was centrifuged at 3000 rpm for 10 min at 4 °C. Plasma (250µl) was then pipetted to microfuge tubes preloaded with 10 µl of 2mM butylated hydroxytoluene (BHT) and frozen at -20 until analysis. Internal standards were added to a vial and dried off before 100µl plasma was added. The samples were acidified with 3M acetate buffer pH 4.8 (50 µl/ml). To hydrolyse the conjugated compounds in the sample, H. *Promatia* extract with β-glucuronidase and sulfatase activity, was used (25 µl/ml). After overnight incubation at 37°C, concetrated HCl (2% v:v) was added and the sample was centrifuged for 10 min at 8000 rpm. The supernatant was added to a solid phase column filled with diatomaceous earth (100 mg/100 µl), which retains the water-soluble compounds. The phenolic acids were eluted with ethyl acetate. The eluate was dried and derivatized by adding 10 µl of acetonitrile and 30 µl of derivatizing agent *N*,*O*-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA). Eight anthocyanin-related phenolic acid metabolites were targeted for quantification: ferulic, isoferulic, caffeic, p-coumaric, syringic, vanillic, 4-OH benzoic and t-cinnamic acids.

6.2.3 Cognitive tasks

Four sensitive and specific cognitive tasks were administered at baseline and at 6 hours to reflect acute change. The interview administered tasks were conducted individually by a trained research assistant. These tasks were selected on the basis of previous research findings (30, 31) which critiqued the application of various cognitive tasks in previous flavonoid interventions and provided recommendations for future studies. Specifically, measures of executive functioning were recommended, however, the specific cognitive domains affected and the tools which are able to measure this change have not yet been elucidated. Consequently the following tasks were selected on the basis of previous research and the fact that they were quick to administer, and challenging but not cognitive draining. A task switching test measured higher executive function by asking participants to alternate between two simple tasks at once (32). The Rey Auditory Verbal Learning test (RAVLT) (33) assessed verbal learning and memory by asking participants to recall a list of words over 5 trials. Pattern and letter comparison tasks (34) assessed speed of processing, asking participants to compare strings of patterns or letters as quickly as possible.

6.2.4 Statistical analysis

Statistical analysis was conducted using SPSS statistical program (V17.0: 2006, SPSS, Inc., Chicago, II, USA). Descriptive analysis was performed for cognition, blood pressure and biomarker variables (mean, SD, range).

Cognitive data were assessed for normality using the Shapiro–Wilk test and found to be normally distributed. Mixed ANOVA with repeated measures determined if differences between age groups or dosage existed for each of the four cognitive tests. Post hoc analyses (Bonferroni correction, p=0.05) were conducted to assess whether outcomes differed between time points for either dose.

Blood pressure data were assessed for normality using the Shapiro–Wilk test and found to be normally distributed. Mixed ANOVA with repeated measures determined if differences between age groups or dosage existed for blood pressure variables (Systolic blood pressure SBP, Diastolic Blood Pressure DBP, Heart Rate HR). Post hoc analyses (Bonferroni correction, p=0.05) were conducted to assess whether outcomes differed between time points.

A large inter-individual variation in baseline levels of biomarkers between individuals (regardless of group) was accounted for by calculating the percentage change (from baseline) at 2 and 6 h post-intervention for each biomarker. These variables were assessed for normality using the Shapiro–Wilk test, showing that all variables were not normally distributed. Variables were transformed using a Log transformation. The percentage change at 2 and 6 h post intervention was compared over time for each dose using repeated measures ANOVA and Bonferroni post hoc analyses.

6.3 Results

Sixteen participants were recruited including 6 young healthy participants, 5 older healthy adults and 5 older adults with dementia (Table 6-1). Three healthy older adults withdrew before the second session. No difference between the three groups at baseline was found for BMI. MNA score differed significantly between groups, but mean values fell within the well-nourished category (MNA>24) for all groups (Table 6-1).

Table 6-1 Subject sample characteristics according to group at baseline

Characteristic Average (SD)	**Young group n=6**	**Older group n=5**	**Dementia group n=5**	**p-value †**
Age in years	21.8 (0.97)	74.1 (7.9)	79.8 (3.6)	<0.001
BMI (kg/m2)	26.3 (4.6)	27.6 (4.6)	29.6 (3.2)	0.189
MNA Score	30 (0)	27.79 (1.0)	25.3 (2.2)	<0.001

† ANOVA for differences between groups

BMI: Body Mass Index, MNA: Mini Nutritional Assessment.

6.3.1 Cognitive tasks

One way ANOVA showed significant differences between all groups for all cognitive tasks at baseline, except the task switching test, where the older and dementia groups performed similarly. No significant differences were found in cognitive performance for the RAVLT, task switching test and pattern or letter comparison tasks, with the exception of the task-switching test in older adults after the single 300ml dose, which was statistically significantly different 6h post cherry juice consumption (p=0.021) (Table 6-2).

6.3.2 Blood pressure

No group effect was observed for change in blood pressure and heart rate over time following administration of either dose so groups were analysed together. A single 300ml dose of cherry juice resulted in a significant reduction at 2h in heart rate (p=0.024) and diastolic blood pressure (p=0.016), and approached significance for systolic blood pressure (p=0.066). A significant quadratic trend was evident. Post Hoc analysis using the Bonferroni correction showed no significant differences between baseline and 6 hours for any of the blood pressure measures. However, a significant difference between baseline and 2h was found for diastolic blood pressure (p=0.02) and heart rate (p=0.032), and between 2h and 6h for systolic (p=0.017) and diastolic (p=0.046) blood pressure, and heart rate (p=0.013) (Figure 6-1). The 3x100ml dose of juice showed a similar but weaker trend and lacked significance.

Table 6-2 Change in performance on cognitive assessments over 6 hours according to group.

	3x100ml serving over 3h	**1x300ml serving**

Assessment	**Baseline** Average (SD)	**Mean difference at 6h**	**Baseline** Average (SD)	**Mean difference at 6h**
Young group	**n=6**		**n=6**	
RAVLT (total)	63.7 (6.7)	+2.0	62.5 (5.6)	+1.5
RAVLT (20m delay)	12.0 (2.5)	+0.5	12.0 (3.2)	+1.3
Task Switching	91.2 (29.9)	-143	78.8 (10.2)	-3.5
Pattern Comparison	21.5 (2.1)	+1.2	21.2 (3.9)	+2.0
Letter Comparison	13.0 (1.9)	+0.67	13.8 (3.3)	-0.33
Older group[a]	**n=2**		**n=5**	
RAVLT (total)	35.0 (2.8)	+2.0	44.6 (5.7)	-4.8
RAVLT (20m delay)	5.0 (1.4)	-0.5	5.8 (2.7)	-0.4
Task Switching	84.6 (3.4)	+0.36	126.6 (28.7)	-21.6 *
Pattern Comparison	14.0 (1.1)	-0.5	12.0 (1.3)	+1.2
Letter Comparison	9.5 (2.1)	-1.0	8.6 (1.5)	+0.8
Dementia group	**n=5**		**n=5**	
RAVLT (total)	25.8 (11.6)	+1.8	25.0 (11.4)	+1.8
RAVLT (20m delay)	0.00 (0)	0	0.00 (0)	0.00 (0)
Task Switching	178.1 (130.1)	+13.4	132.1 (109.7)	-3.5
Pattern Comparison	9.2 (5.6)	-0.6	8.2 (4.8)	-0.8
Letter Comparison	4.0 (2.7)	+1.2	4.6 (3.2)	+0.6

RAVLT (total) and (20m delay) = number of words recalled

Task switching = seconds taken to complete task

Pattern and letter comparison = number of correct answers in 1 minute

* p=<0.05

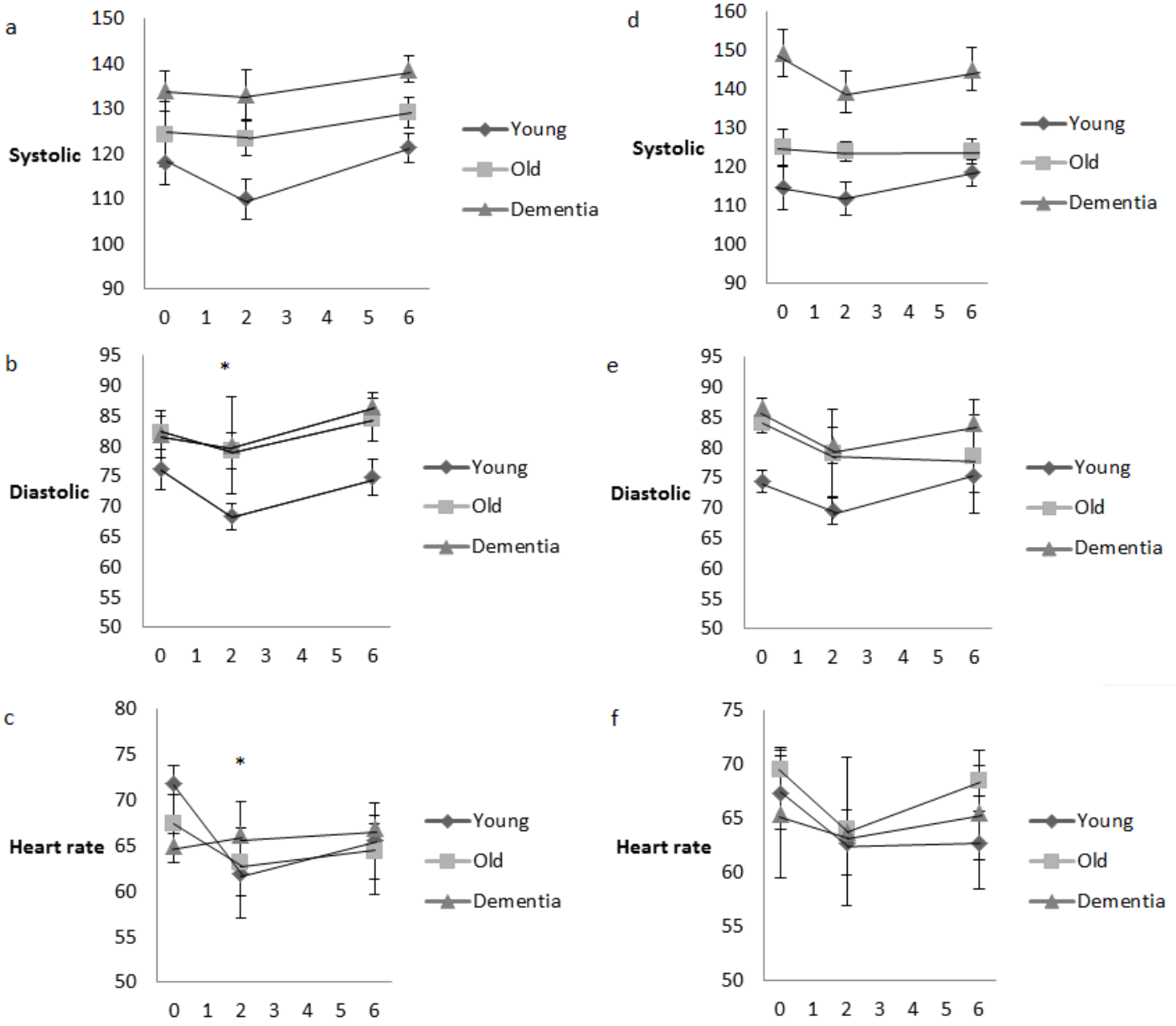

Figure 6-1. Acute change in blood pressure and heart rate over 6 hours after either a single 300ml dose of cherry juice (a, b, c) or 3 x 100 ml doses over 3 hours (d, e, f)

6.3.3 Phenolic acid biomarkers

Eight phenolic acids were detected in all plasma samples, with the exception of vanillic and t-cinnamic acid for older adults at the 3x100ml dose visit (n=2). On both occasions, baseline concentrations of plasma phenolic acids were highly variable and differed between groups, except for syringic and vanillic acid (Table 6-3). A single 300ml serving of cherry juice resulted

in large changes in all plasma phenolic acids at 2 and 6h post intervention (Figure 6-2), particularly caffeic acid. The 3x100ml dose of the juice administered over 3 hours resulted in smaller plasma phenolic acid fluctuations, and in some individuals, plasma concentrations declined from baseline. No significant difference between the absolute plasma phenolic concentrations over time was evident for either serving or between groups. Percentage change in plasma phenolic acid concentration at 2h and 6h was calculated for each individual, relative to the baseline concentration, to mediate individual inter-variability. The mean percentage change was calculated for each group at 2h and 6h from baseline (Figure 6-2).

Table 6-3 Mean baseline concentrations of plasma phenolic acids according to group (average of two baseline measures)

Group Mean +	**Ferulic Acid***	**Isoferulic Acid ***	**Caffeic Acid ***	**p-coumaric Acid ***	**Syringic Acid**	**Vanillic Acid**	**4-OH-Benzoic Acid***	**t-cinnamic Acid ***
Young	0.20	0.21	0.084	0.019	0.041	1.047	4.19	0.31
Older	0.13	0.51	0.0054	0.04	0.027	0.94	8.37	0.059
Dementia	0.23	0.61	0.027	0.032	0.018	0.70	7.13	0.081

+ nm/ml, *p<0.001 One way ANOVA

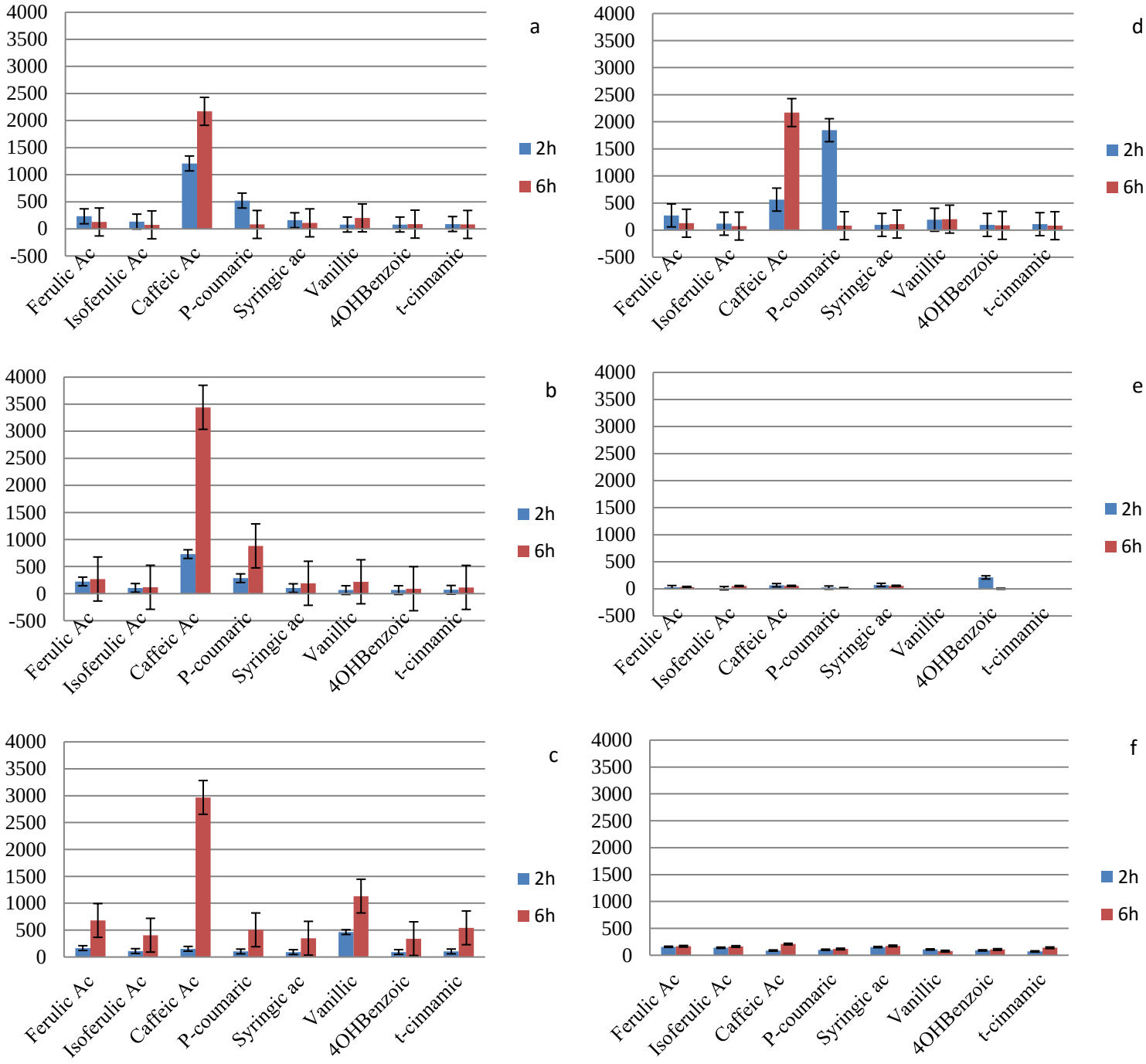

Figure 6-2. Percentage change in plasma phenolic and aromatic acid concentration at 2 and 6h post intervention; a) young b) old c) dementia for 1x300ml dose and d) young e) old f) dementia 3x100ml dose over 3h

6.4 Discussion

This exploratory study developed methodology to examine the acute dose dependent impact of flavonoids from cherries on cognition and blood pressure, in young people, older adults and older adults with mild to moderate dementia. Evaluating the intervention highlights several positive and negative characteristics of its design. The intervention was well tolerated, with no adverse reactions to the juice. The attrition of three participants in this small cohort was related to study burden and length of testing. The cross-over design appears to appropriately account for the high intra-individual variations in the metabolism and absorption of flavonoids. However, there are limitations associated with this study, including the small number of participants, which restricts our interpretation of the comparison between the groups. Also, the lack of a control group in this pilot study hinders the inferential interpretation of these results. However, it was the aim of this study to describe the differences between two dose-timing administrations. The results should be interpreted with caution.

6.4.1 Cognitive outcomes

Data from this pilot study indicates that a feasible serving of cherry juice, regardless of dose-timing, does not appear to influence acute cognition. The significant difference over 6h for the older group between doses for the task-switching test may be attributed to the attrition of subjects in that group. While the cognitive assessment tasks included in this study have previously been shown to be sensitive measures for nutrition interventions (30, 35) this pilot study is underpowered to detect changes in cognition. However, the cognitive assessment battery may be useful for application in a larger study as they were easily applied, interpreted and analysed in all patient groups in this study. The single 300ml dose of cherry juice provided in our study, and consequent dose of anthocyanins (≈55mg) is lower than doses of flavonoids provided through a variety of foods in other studies (36) may also explain the lack of effect. Other limitations relate to the lengthy testing sessions, leading to fatigue or lack of motivation, which may implicate performance in the cognitive tasks (37). Different versions of each task were administered at each time-point; however, trends for improvements in cognition may be attributed to a learned practice effect (38). Additionally, the influence of study timing should be further considered, whereby blood pressure may be influenced (potentially increased) by the timing of cognitive tasks. Therefore, future research should be informed by recent reviews of sensitive and specific cognitive tests for nutrition interventions (31, 35). Additionally, it is

important to link the hypothesised physiological effects more closely with application of cognitive tasks. A cognitive task administered 2h post intervention would reflect the immediate absorption of intact flavonoids in the small intestine as well as avoiding testing fatigue; however the sensitivity of cognitive tests needs to be improved.

6.4.2 Blood pressure

A reduction in acute blood pressure and heart rate measurements were seen after a single 300ml dose of cherry juice after 2 hours in all three groups similarly, before returning to baseline levels at 6h (Figure 6-1). The reduction of blood pressure and heart rate measurements at 2 hours is supported by data from other acute anthocyanin feeding studies (39), whereby anthocyanins reportedly reach the circulatory system within 0.25–2hrs and are excreted by 4hrs post-prandially. However the same amount provided in three smaller doses over a 3 hour period did not provide the same effect. This highlights potential dose-dependency and suggests that a minimum threshold of ingestion or uptake may need to be reached in order to induce bioactive effects. However, replication of these results in a larger sample is required. Ambulatory BLOOD PRESSURE monitoring may be more precise to accurately explore blood pressure fluctuations over 6 hours and could be utilised to measure the extended effects of flavonoid rich foods. Our exploratory study did not investigate possible mechanisms for blood pressure lowering effects and as fluctuations in plasma phenolic acids generally peaked at 6h post intervention, reflecting their uptake in the colon, they are unlikely to have influenced blood pressure changes. Given the large body of existing research demonstrating reductions of inflammatory and oxidative stress markers and increases to endothelial function associated with anthocyanin consumption, these mechanisms should be primary considerations in future studies (23).

6.4.3 Plasma biomarkers

After correcting for baseline plasma concentrations to account for individual inter-variation in flavonoid metabolism and absorption (40), the percentage change in plasma phenolic acids increases at 2h and 6h post intervention for both the single 300ml and 3x100ml doses of juice. However, the relative increase for the single 300ml serving of juice was greater than the single 300ml serving, indicating a larger uptake of cherry related phenolic acids after the single 300ml serving. Older adults and dementia patients showed a lessened response to the 3x100ml serving

over 3 hours when compared to younger adults, which may indicate a reduced ability to absorb and metabolise flavonoids in this group. This hypothesis requires further investigation on a larger and more controlled scale alongside studies of dose-timing relationships for flavonoid consumption.

The increase in plasma phenolic acids at 2h post intervention are likely to reflect the absorption of flavonoids and phenolic acids from cherry juice in the small intestine, as well as smaller absorption from the stomach (41). The increases shown at 6h post intervention are more likely to reflect absorption of flavonoid metabolites from the colon (41). The eight phenolic acids measured in the study are related with anthocyanin metabolism and should reflect the metabolism and uptake of anthocyanins in cherry juice. However, due to the wide spanning nature of phenolic acids in foods and diet, and endogenous phenolic acids generated by human metabolism that the phenolic acids may have come from other sources, such as the metabolism of the standardised breakfast. There was no statistically significant difference in plasma phenolic acid concentration between groups or over time for either serving, which is largely reflective of their extreme variability in this small cohort. Due to the high intra-individual variation of flavonoid metabolism and bioavailability, the fact that phenolic acids are influenced by factors other than intake, such as bioavailability and genetic factors, the combined use of plasma biomarkers and dietary intake estimates may be the best choice in future studies (42).

The methodology piloted in this study provides an indication of both positive and negative aspects of its design. An important aspect of the research design is the feasibility of the intervention in terms of translation into practice. Small changes to the study protocol will likely improve the accuracy of larger trials. This pilot study indicates that acute intake of flavonoids in a feasible serving of cherry juice does not change cognitive performance, but may reduce blood pressure. A lack of effect for the three small servings of juice over 3 hours suggests dose-interval administration may influence the bioactive effect of flavonoids, which require further investigation.

6.4.4 Conclusion

The findings from this pilot study suggest that further study of cherry juice and dose response variations from consumption on vascular and cognitive outcomes is warranted in light of the evaluated methodology. Further application of the phenolic acid biomarkers in a larger sample should be conducted to assess their ability to reflect flavonoid intake, and confirm the absorption and metabolism of cherry-related flavonoids.

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CHAPTER 7: CONSUMPTION OF ANTHOCYANIN-RICH CHERRY JUICE FOR 12 WEEKS IMPROVES MEMORY AND COGNITION IN OLDER ADULTS WITH MILD TO MODERATE DEMENTIA

The chapter describes a 12-week randomised controlled trial that aimed to assess whether daily consumption of anthocyanin-rich cherry juice changed cognitive function in older adults with dementia. Blood pressure and anti-inflammatory effects were examined as secondary outcomes. Older adults (+70y) with mild to moderate dementia (n=49) consumed 200ml/day of either a cherry juice or a control juice with negligible anthocyanin content. Blood pressure and inflammatory markers (CRP, IL-6) were measured at 6 and 12 weeks. Improvements in verbal fluency (P=0.014), short term memory (P=0.014) and long term memory (P=<0.001) were found in the cherry juice group. A significant reduction in systolic (P=0.038) blood pressure and a trend for diastolic (P=0.160) blood pressure reduction was evident in the intervention group relative to the control. Markers of inflammation (CRP and IL-6) were not altered. Inclusion of an anthocyanin-rich beverage may be a practical and feasible way to improve total anthocyanin consumption in older adults with mild to moderate dementia, with potential to improve specific cognitive outcomes.

This chapter has been published in the European Journal of Nutrition:
Appendix B

Kent, K, Charlton K, Roodenrys S, Batterham M, Potter J, Traynor V, Gilbert H, Morgan O, Richards R, 2015. Consumption of anthocyanin-rich cherry juice for 12 weeks improves memory and cognition in older adults with mild to moderate dementia. European Journal of Nutrition DOI: 10.1007/s00394-015-1083-y

Analysis of the baseline dietary and cognitive data has published in the Journal of Aging: Research and Clinical Practice:
Appendix D

Kent, K, Roodenry, S, Charlton, KE, 2016. Dietary flavonoid intake and cognitive performance in older adults with Alzheimer's type dementia. Journal of Aging: Research and Clinical Practice, vol. 5.

This data was also presented at several National conferences:

Caldwell*, K., Charlton, K., Roodenrys, S., Batterham, M., Potter, J., Richards, R., Gilbert, H. & Morgan, O. 2014. The impact of fruit flavonoids from cherries on memory and cognition in older adults with mild to moderate dementia. Abstracts of HSA 2014 Annual Scientific Meeting (pp. 1-1). Australia: HSA. Abstract of a presentation that was present at the NSA 2014 Annual Scientific Meeting, 26-28 November, Hobart, Australia.

Caldwell*, K. & Charlton, K. 2013. Dietary intake and major sources of flavonoids in older Australians with Alzheimer's type dementia. Enabling Active Ageing Conference Proceedings: The 12th National Conference of Emerging Researchers in Ageing (pp. 44-44). 25 and 26 of November 2013, Sydney, Australia. Australia: Emerging Researchers in Ageing Australia.

Caldwell*, K., Charlton, K. E. & Roodenrys, S. J. 2013. Estimation of dietary flavonoid intake and cognitive performance in older adults with Alzheimer's type dementia. Australasian Medical Journal, 6 (11), 612. Abstract presented at The Nutrition Society of Australia and Nutrition Society of New Zealand 2013 Joint Annual Scientific Meeting, 4-6 December 2013, Brisbane, Australia

KK was responsible for organisation and leading of the study, data collection and analysis and preparation of the manuscript. KEC and SR were responsible for the design of the study and analysis and critical revisions of the manuscript. MB contributed advice regarding the statistical analysis of the study. JP and VT advised and facilitated patient recruitment. RR, HG and OM contributed to critical discussions regarding the cognitive tests used and baseline data collection.

7

7.1 Introduction

Plant-based foods form an integral component of the human diet and their consumption is consistently linked to the maintenance of health and the prevention of a vast array of diseases (1). A growing body of evidence has shown that phytochemicals, non-nutritive bioactive compounds, contribute to the antioxidant activity of individual fruits and vegetables and are consequently credited with the observed health benefits (2). Flavonoids are a class of polyphenols that have been studied intensively and are categorised into six major classes: anthocyanins, flavanols, flavanones, flavones, flavonols, and isoflavones (3). Flavonoids are found in particularly high concentrations in fruits and vegetables, wine, tea and cocoa (4). The consumption of flavonoids has been associated with a reduction in risk for cardiovascular diseases and some cancers (5, 6) and more recently there has been attention directed to their potential to protect against neurodegenerative diseases and improve cognitive performance in older adults (7).

The sub-groups of flavanols, anthocyanins and flavanones have been shown to be the most beneficial of the flavonoid family in terms of neuro-protection (8). Specifically, literature investigating the impact of fruit flavonoids on cognitive and physical outcomes is predominately pre-clinical (9) and while promising, remains inconclusive. Much of the food-based research has focused on flavonoid-rich blueberries and strawberries (9). The biological actions of flavonoids on cognitive function have been attributed to a number of hypothesized mechanisms. Their antioxidant actions assist to scavenge free radicals in the brain (8), while their neuro-protective effects include protection of vulnerable neurons against inflammation, enhancement of existing neuronal function, increased blood flow to the brain and neurogenesis initiation in areas of the brain that are associated with cognition (10).

Preliminary dietary supplementation studies have shown that blueberries (11) and concord grapes (12, 13) improve aspects of memory in older adults over a 12 and 16 week intervention. Cherries, both the sweet and tart varieties, are a rich source of anthocyanins and to a lesser extent also contain flavan-3-ols and flavonols (14). Cherries have been found to lower

inflammation and scavenge nitrous oxide radicals within the body (15). However the potential of cherry flavonoids to influence cognitive function has not been investigated, despite them being a commonly consumed fruit in Australia (4) and other countries.

Research to date has investigated the cognitive enhancing effects of flavonoid-rich foods in participants with both normal cognitive function (16) and people with mild cognitive impairment (11-13) but effects in dementia patients remains under researched (17). In light of projections indicating rapid increases in the prevalence of the dementia (18) and in the absence of successful treatments, alternative measures to slow the development and progression of dementia are imperative.

A randomised clinical trial was conducted to assess changes in cognitive function in older adults with mild to moderate dementia after daily consumption of a feasible serving of anthocyanin-rich cherry juice over 12 weeks. Secondary outcomes included anti-inflammatory effects, changes in functional and physical ability and mood.

7.2 Methods

7.2.1 Participants

Participants were recruited from a geriatric outpatient dementia clinic and residential aged care facilities in the Illawarra region of New South Wales, Australia. Participants were aged 70 years or older and had been diagnosed as having mild to moderate dementia Alzheimer's type, as confirmed by the consultant geriatrician responsible for their clinical management (JP). Exclusion criteria include non-English speaking, uncontrolled hypertension, uncontrolled diabetes, any other unstable physical or mental health condition or dysphagia. Written informed consent was obtained. For participants with cognitive impairment deemed by their geriatrician to be too severe to provide informed consent, consent was provided by family carers or guardians. Consenting participants (Table 7-1) were randomised to a treatment group and randomised by computer-aided block randomisation, conducted by a statistician that was independent to the research interface (MB) (Figure 7-1). All other researchers were blinded to

the grouping. This study was approved by the University of Wollongong Human Research Ethics Committee and the Illawarra Shoalhaven Local Health Districts ethics committee (HE11/175). This study was registered as a clinical trial through the Australian New Zealand Clinical Trials Registry (ANZCTR) and was allocated the ACTRN: ACTRN12614001298606.

7.2.2 Study Procedure

Over 12 weeks participants received 200ml/day of cherry juice (intervention arm) or 200ml/day of commercially prepared apple juice (control arm). Juice was delivered weekly, by a research assistant not involved in data collection, to the homes of participants, chilled in 1L plastic bottles. Instructions were provided to participants and carers for the juice to be consumed daily, at any time in one sitting, using a cup provided that indicated 200ml with a fill line. A weekly calendar was provided with the juice, with daily check boxes ticked when the juice was consumed. The sweet, Bing cherry juice was produced using a novel method that aims to retain the phenolic bioactives, developed by a research company (Agritechnology) based in Orange, NSW, Australia. The high antioxidant activity and anthocyanin content of cherries is well retained in the juice which had a high Oxygen Radical Absorbance Capacity (3200 µmol Trolox Equivalents/g) and contained 69 mg red pigments (anthocyanins) per 100g of juice as determined by HPLC (19) (Table 7-2). The apple juice was provided by Appledale, based in Orange, NSW, Australia (Table 7-2). Nutritional content of the intervention and control juices are shown in Table 7-2. A serving size of 200ml per day was determined as being a feasible quantity that could be consumed by an older adult with mild-moderate dementia, and was chosen in the absence of empirical research that highlights a dose requirement for anthocyanin intake and cognitive outcomes. The serving size provided a more feasible serving of juice than has been used in previous similar studies, where the quantity of anthocyanin-rich beverages provided may exceed the amount that can be feasibly achieved over the long term, especially by older adults living with a neurodegenerative disease (11, 13). No change to regular diet was advised.

Table 7-1 Baseline characteristics of participants in the study according to group

Characteristic	Control group n=25, mean ± SD	Intervention group n=24, mean ± SD
Age (years)	80.6 ± 6.6	78.9 ± 5.2
BMI (kg/m^2)	26.6 ± 3.5	25.7 ± 3.4
Current smokers (count)	1 (4%)	0 (0%)
Previous smokers (count)	10 (40%)	7 (29%)
Hand grip strength (kg)	52.1 ± 16.4	62.5 ± 16.1*
Mid arm circumference (cm)	26.9 ± 4.5	27.2 ± 4.1
Calf circumference	33.4 ± 3.7	34.9 ± 3.4
Education (years)	17.5 ± 3.2	18.2 ± 2.4
Mini Nutritional Assessment	23.4 ± 3.5	25.6 ± 2.8*
Instrumental Activities of Daily Living	5.7 ± 2.4	7.5 ± 0.8*
Total flavonoid intake (mg/day)	429.8 ± 384.2	599.0 ± 352.2
Flavonol intake (mg/day)	20.0 ± 13.2	32.9 ± 26.5
Flavone intake (mg/day)	0.3 ± 0.3	1.2 ± 1.7
Flavanone intake (mg/day)*	8.8 ± 16.4	11.7 ± 17.9*
Flavon-3-ol intake (mg/day)*	382.9 ± 379.6	518.9 ± 338.9*
Anthocyanin intake (mg/day)	13.1 ± 29.7	34.2 ± 55.6*

*P<0.05, for differences between groups.

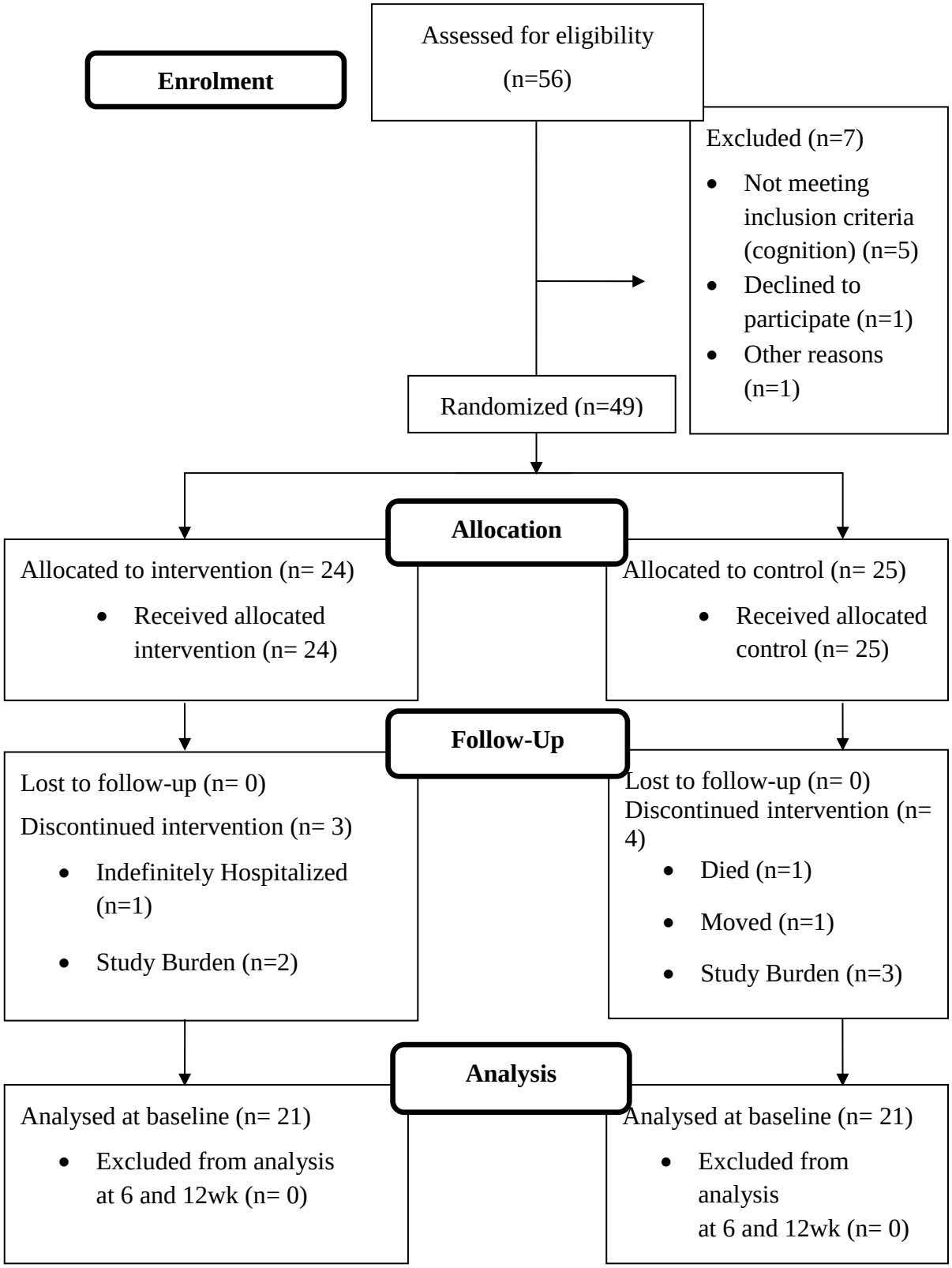

Figure 7-1 CONSORT flow diagram of enrolment, allocation, follow up and analysis.

Table 7-2 Nutritional properties of intervention and control juice

	Cherry Juice (100ml)	**Apple Juice** (100ml)
Energy	395kJ	180kJ
Protein	1.5g	0.1g
Fat Total	<0.2g	0.1g
Saturated	<0.2g	0.1g
Carbohydrate Total	21.0g	10.5g
Sugars	16.0g	9.3g
Dietary fibre	0.7g	0.0g
Sodium	<1mg	1.0mg
Oxygen Radial Absorbance Capacity	3200 µmol TE [a]/ g	15.55 µmol TE[a]/ g
Red Pigment (Anthocyanin) content	69 mg [b]	0.02mg [c]

[a] Trolox Equivalent

[b] Iland Method (19).

[c] USDA database for the flavonoid content of selected foods (release 3.1) (14).

7.2.3 Measurements

Outcome variables were measured at three time points: baseline, 6 weeks and 12 weeks with all interviews conducted at the same time of the day (am). At baseline and 12 weeks participants arrived fasting and a blood sample was collected by a trained phlebotomist. A standardised breakfast was offered to participants (cereal, milk, banana, tea and/or coffee) and they were instructed to consume the meal ad-libitum, before the remainder of the interview was conducted. At 6 weeks, participants arrived at the clinic after having consumed their usual breakfast at home.

At each interview, a questionnaire was administered by a single researcher, with the assistance of a guardian or carer, where appropriate, reporting selected demographic characteristics, tobacco use, and consumption of alcohol, supplements and medications. Nutritional status was assessed using an interviewer-administered Mini Nutritional Assessment (MNA) (20) and dietary intake, including flavonoid intake, was assessed using a 24hr dietary recall method,

with the assistance of a carer or guardian. Dietary records were entered into the FoodWorks dietary assessment programme to assess nutritional parameters (Xyris Software, Highgate Hill, QLD, Australia, Version 5, 2007). To estimate usual flavonoid intake, dietary records were cross-referenced with the 2013 USDA database for the flavonoid content of selected foods release 3.1 (14). Resting BP and heart rate were measured using an Omron HEM7200 Deluxe Automatic BP Monitor, while seated, in triplicate and averaged. To monitor physical outcomes over 12 weeks, anthropometric measurements included height, weight, and mid arm and mid-calf circumference. Lawton's Instrumental Activities of Daily Living Scale (IADL) (21) determined functional impairment and hand grip strength was assessed using a digital Jamar handgrip dynamometer (Lafayette Instruments, Indiana, USA). The blood sample assessed changes in markers of inflammation (C-reactive protein (CRP) and Interleukin-6 (IL-6) and plasma vitamin C levels. Blood samples were prepared and stored at -80^{o}C for batch analysis by an independent laboratory where IL6 was measured by High Sensitivity cytokine panel (millipore), CRP was measured by hsCRP (Kamiya), and Vitamin C by an in-house method.

7.2.4 Outcome assessment

Cognitive function was assessed using a battery of seven cognitive tests, including the Rey Auditory Verbal Learning Test (RAVLT) (22, 23) , the self-ordered pointing task (SOPT)(24), the Boston naming test (25), the trail making test (TMT) (26), digit span backwards task (27) and category and letter verbal fluency (28). The RAVLT measured verbal learning and memory by the participant learning a list of words over presentation-test and delayed-test trials. The SOPT is a test of working memory, and the Boston naming test assessed confrontational naming, related to semantic memory. The TMT A and B evaluated executive function and the relative difference between B and A was used as the measure. Digit span backwards task examined short-term memory storage and executive control processes by requiring participants to repeat a series of numbers in the reverse order they were given. Category and letter verbal fluency measured executive function, requiring participants to recite as many words as possible that belonged to the category "animals" or began with the letter "F" and "A". The geriatric depression scale (GDS) (29) assessed mood state. The battery of cognitive assessments was selected on the basis of previous research findings (30) and the suitability of each assessment as applied in the dementia cohort. Each task had been previously validated and shown to be a sensitive and specific task at measuring the appropriate cognitive domain.

7.2.5 Statistical Analysis

Statistical analysis was conducted using SPSS statistical program (V17.0: 2006, SPSS, Inc., Chicago, II, USA). Analysis was completed using the full cohort that completed each of the time points. Continuous data are described as the mean and standard deviation (SD) as appropriate. Non-normally distributed variables were transformed and treated as normally distributed. Baseline differences between the intervention and control group's characteristics and nutrition information were analysed by unpaired t-tests for continuous variables. A 2-way repeated measures ANOVA analysed the effect of time, treatment, and time*treatment interactions for blood pressure measurements at 6 and 12 weeks. Analysis of covariance (ANCOVA) using the baseline data as a covariate was used to analyse the group effect at 6 and 12 weeks for all cognitive tasks, to isolate the effect of the intervention while controlling for group differences at baseline. Repeated measures ANOVA assessed differences in markers of inflammation and vitamin C at baseline and 12 weeks. A *p* value less than or equal to 0.05 was considered to indicate statistical significance. Eta-squared (η^2) values were calculated to indicate the strength of the main effect. The Cohen's f effect size estimates were characterised as small (0·10), medium (0·25) and large (0·40) (31).

7.3 Results

Forty-nine participants (24 female, 25 male) were recruited and seven participants withdrew from the study (2 indefinitely hospitalised, 4 due to study burden and 1 moved away). Participants were randomised into the control group (n=25) and intervention group (n=24) (Figure 7-1).

7.3.1 Baseline:

No differences between groups were found for age, BMI and anthropometrics, years of education or total flavonoid intake at baseline. Significant differences at baseline were found between groups for measures of malnutrition, activities of daily living, and hand grip strength.

Differences in habitual intake of several flavonoid subclasses was found between the groups, according to a 24h recall administered at baseline (Table 7-1), with the intervention group

consuming more flavonoids than control subjects. Mean total flavonoid intake was estimated as 510mg/day. Black tea (80%) was the most significant dietary source of total flavonoids followed by green tea (7.5%), red wine (4.5%), apples (1.7%) and oranges (1.6%) with their respective fruit juices. Flavonols contributed 5.15% of total flavonoid intake. Dominant sources included black and green tea, onion, broccoli and apples. Flavones contributed the smallest percentage (0.15%) with the major source being parsley. Total flavanone intake provided 2% with major sources including oranges and orange juice, and lemons. Flavon-3-ols contributed 88.1% of total intake, with black tea as the major source and wine and apples contributing somewhat. Anthocyanins (4.6%) were provided by red wine, red grapes and bananas.

At baseline, the only significant difference between the groups for nutrient intake was for carbohydrates (p=0.023) and caffeine intake (p=0.03), with the intervention group with a higher intake than the control.

7.3.2 Post-intervention:

A trend for improvement in most of the cognitive tasks is evident as shown by the mean difference between baseline and 12 weeks for the intervention group only (Table 7-3). Analysis of covariance showed significant improvement in cognitive performance in the intervention group relative to the control (Figure 7-2), at 6 and 12 weeks for the Category Verbal Fluency task (p=0.014) , RAVLT total (p=0.014) , RAVLT delayed recall (p=0.005) and RAVLT 20 minute delayed recall (p=<0.001) tasks. The effect sizes for Category Fluency (η^2 = 0·711), RAVLT total (η^2 = 0.713), RAVLT delayed recall (η^2 = 0.433) were large and the effect size for RAVLT 20 minute delayed recall (η^2 = 0.242) was moderate. No significant improvements from baseline were found for cognitive performance tasks in the control group.

Table 7-3 Mean scores for cognitive performance and mood by group [a]

	Control group			Intervention group		
	Baseline n=25	12 weeks n=21	Mean Difference	Baseline n=24	12 weeks n=21	Mean Difference
Letter Verbal Fluency (executive function)	13.1 ± 7.5	13.1 ± 7.9	0.015 ± 0.4	18.9 ± 11.0	19.0 ± 10.3	0.13 ± 0.7
Category Verbal Fluency (executive function)	8.4 ± 4.5	8.3 ± 4.7	-0.1 ± 0.19	11.9 ± 4.5	13.4 ± 5.1	1.9 ± 0.17
RAVLT total (I-V) (learning and memory)	19.3 ± 9.2	17.5 ± 13.1	-1.8 ± 3.85	25.5 ± 10.6	29.1 ± 11.5	3.9 ± 0.88
RAVLT delayed recall (memory)	1.2 ± 1.9	1.4 ± 2.2	0.2 ± 0.4	2.3 ± 2.6	3.8 ± 2.9	1.6 ± 0.4
RAVLT 20m delayed recall (memory)	0.72 ± 1.2	0.7 ± 1.2	0 ± 0.07	0.6 ± 1.0	2.3 ± 2.6	1.6 ± 1.4
Trail making task [b] (executive functioning)	129.8 ± 81.1	128.6 ± 85.0	-1.2 ± 4.0	125.1 ± 65.0	101.9 ± 67.3	-23.2 ± 2.32
Self-ordered pointing task (working memory)	1.3 ± 1.3	1.6 ± 1.8	0.3 ± 0.5	0.6 ± 1.0	0.6 ± 0.8	0.0 ± -0.2
Digit span backwards task (short-term memory)	2.3 ± 1.1	2.8 ± 1.1	0.5 ± 0	3.0 ± 1.0	3.4 ± 1.2	0.4 ± 0.2
Boston naming task (semantic memory)	33.1 ± 15.5	31.9 ± 13.6	-1.2 ± -1.8	40.6 ± 13.1	40.0 ± 13.6	-0.5 ± 0.5
Geriatric Depression Scale [b] (mood)	7.3 ± 4.7	6.9 ± 3.5	-0.4 ± -1.2	6.9 ± 4.5	6.3 ± 4.3	-0.6 ± -0.2

[a] Data are given as mean ± SD. Baseline refers to measures obtained at the pre-intervention assessment. Final refers to measures obtained during the final week of the intervention. Difference = final score (mean) less baseline score (mean).
[b] A negative score is indicative of improved outcomes. Otherwise, a positive difference in score indicates improved outcomes.

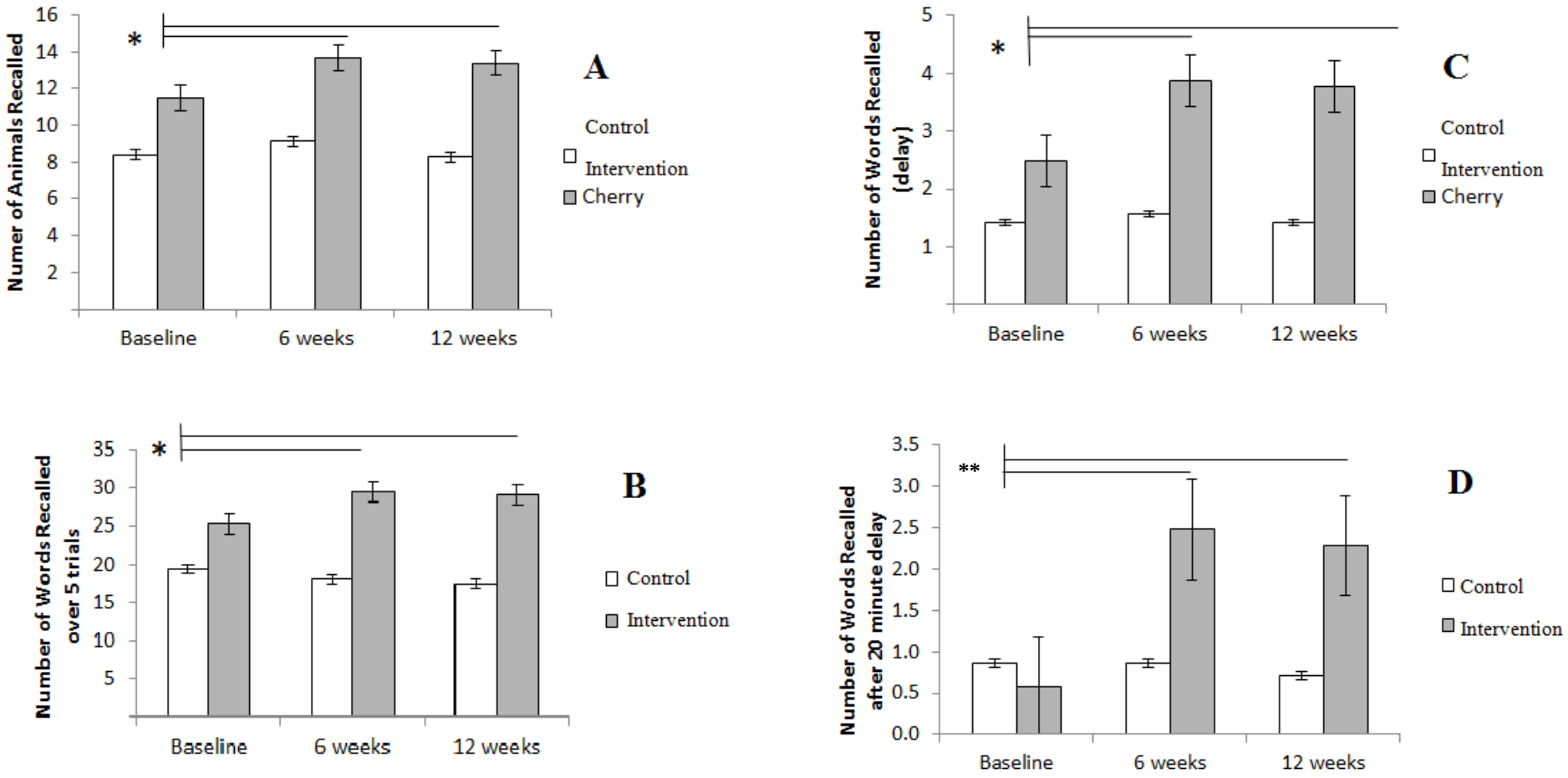

Figure 7-2 Significant changes in category fluency (A), RAVLT total (B), RAVLT delayed recall (C) and RAVLT – 20minute delayed recall (D) at 6 and 12 weeks post intervention
*p=<0.05**p=<0.001

Table 7-4 Blood pressure and heart rate measurements according to group at baseline, 6 and 12 weeks [a]

	Control group			**Intervention group**		
	Baseline n=25	6 weeks n=21	12 weeks n=21	Baseline n=24	6 weeks n=21	12 weeks n=21
Systolic BP*	140 ± 19.7	138.5 ± 12.3	137.0 ± 10.1	138.2 ± 16.4	133.7 ± 9.9	130.5 ± 12.2
Diastolic BP	80.6 ± 9.8	81.0 ± 8.0	81.3 ± 11.6	78.6 ± 11.7	77.0 ± 9.9	77.0 ± 12.6
Heart Rate	70.2 ± 10.2	70.2 ± 11.1	74.2 ± 11.8	67.9 ± 10.7	66.0 ± 7.2	67.5 ± 7.9

*p=<0.05

Data are given as mean (mmHg) ± SD.

[a]Baseline refers to measures obtained at the pre-intervention assessment. 6 weeks and 12 weeks refers to measures obtained at these time points during the intervention.

Repeated measures ANOVA, with the baseline as a covariate, showed a significant difference in systolic blood pressure (p=0.038) at 6 and 12 weeks post baseline, with a similar trend evident for diastolic blood pressure (p=0.160) in the intervention group relative to the control (Table 7-4). No significant differences in blood pressure are evident for the control group.

At follow up, there were no significant between-group differences in nutrient intake or for change in nutrient intake from baseline.

Repeated Measures ANOVA showed no significant difference between the intervention and control groups for serum vitamin C, IL-6 or CRP at baseline or 12 weeks (Table 7-5). There were no improvements in serum vitamin C levels after 12 weeks in either group. Mean levels of plasma CRP and IL-6 were not significantly different after the intervention.

Table 7-5 Serum vitamin C and inflammatory markers at baseline and 12 weeks

	Control group n=25		**Intervention group** n=24	
	Baseline	12 weeks	Baseline	12 weeks
IL6 (pg/mL)[a]	4.3±2.9	6.3±5.8	3.7±1.8	3.8±3.5
hsCRP (mg/L)[b]	2.0±2.5	2.0±2.3	1.6±1.5	1.7±1.8
Vitamin C (uM)	19.3±13.1	13.6±8.2	18.2±10.6	13.3±11.0

[a]IL-6 Interleukin-6; [b]hsCRP C-Reactive Protein

Data are given as mean ± SD

7.4 Discussion

This study found that daily consumption of a feasible serving of anthocyanin-rich cherry juice for 12 weeks improved cognitive performance across almost all tasks in older adults with mild to moderate dementia. To our knowledge, this is the first controlled human trial examining cognitive and physical responses to a dietary intervention involving sweet cherries as a source of flavonoids and anthocyanins.

Our study suggests that provision of anthocyanins through an achievable and acceptable daily quantity of sweet cherry juice over 12 weeks has benefit for cognitive function in older adults with Alzheimer's type dementia. Trends are evident, showing improvements in cognitive performance across all tasks with regular cherry juice consumption. Statistically significant improvements are seen for category verbal fluency and tasks relating to verbal learning and memory (Figure 7-2). The moderate and large effect sizes seen for the cognitive tasks highlight the clinical relevance of these cognitive improvements. These findings are consistent with those of recent human and animal studies showing improvement in cognitive performance in these domains with dietary supplementation with other anthocyanin-rich food sources (11-13, 32-34). Although, we have previously shown that intake of cherry juice does not impact acute cognition over 6h (35). The ability of flavonoids to modulate Alzheimer's disease progression is still poorly understood (17). As explored by the hypothesis in the current study, flavonoids may be more likely to hinder both normal and disease-related losses in cognitive performance through their actions on the brain's cellular and molecular architecture of memory, rather than halt disease progression.

Some nutritional differences existed between the intervention and control juices (Table 7-2). As intended, the ORAC measure of oxidative capacity (3200 vs 15.55 µmol TE/g) and the red pigment (anthocyanin) content (690mg/L vs 0.2mg/L) is much higher in the experimental cherry juice compared to the control apple juice. Apple juice was chosen as a control juice as it represents a commonly consumed beverage in older adults, but that has negligible anthocyanin. Despite literature indicating beneficial effects of apples and apple flavonoids for health outcomes, the juice utilised in the experiment was processed in a way that likely degraded the flavonoid content. This important aspect of the research design is relevant to translation of the findings into dietary messages. However, it is important for future work to investigate whether dose-response effects are evident in such nutrition interventions.

Despite randomisation, differences in several anthropometric and nutritional factors existed between the intervention and control group at baseline (Table 7-1). We attempted to mediate this factor statistically, by applying baseline as a covariate in the analyses. However, these differences in general health and baseline cognition may

have influenced the outcomes seen for the intervention group. The estimation of total flavonoid intake and intake of specific flavonoid subclasses at baseline revealed large variability between subjects. This can be principally attributed to variations in tea (confirmed by significant differences in caffeine intake between groups), wine and other fruit juice consumption, which were the main sources of flavonoids in this sample. Total intake and major sources of flavonoids of participants at baseline was similar to that reported for Australians aged 65+ years (510 vs 575 vs 683mg/day respectively) (4, 36, 37). The higher anthocyanin intake in our sample, compared to national estimates, may be explained by a relatively high consumption of red wine in the study group. The addition of cherry juice to the diet of the intervention group provided them with an additional 138mg anthocyanin/day, which increased their total anthocyanin intake to 46 times greater than the national estimate for the daily intake of adults aged 65+y (3.02mg/day). In the absence of a Nutrient Reference Value (NRV) for flavonoids (38), this magnitude of increase in consumption can be considered to be a significant increase above habitual intake levels for this age group.

Secondary outcome measures included anti-inflammatory effects, changes in functional and physical ability and depressive symptomatology. There was a significant reduction in systolic blood pressure and a trend for diastolic blood pressure in the intervention group relative to the control; however the study was not adequately powered to detect blood pressure changes. Previous intervention trials have hypothesised that the mechanisms relating to the improvements seen in cognitive performance after anthocyanin-rich food supplementation are due to a reduction in inflammatory markers, resulting in a blood pressure-lowering effect (13). Our study showed that markers of inflammation were not significantly altered after cherry juice supplementation (Table 7-5) and remained within the clinically normal ranges (CRP <5mg/mL; IL-6 <10pg/mL). This finding suggests that the bioactive components provided by the cherry juice may provide other benefits, such as stimulating an up-regulation of signalling cascades in areas of the brain relating to memory (8). Additionally, as Alzheimer's disease is associated with progressive and chronic inflammation (39), the disease pathology may mask any potential anti-inflammatory effects provided by the cherry juice.

Surprisingly, serum vitamin C levels decreased in both groups at 12 weeks of follow up (Table 7-5). This difference cannot be explained through changes in dietary patterns over the study duration as vitamin C intake did not differ according to 24h diet recall data. Additionally, self-reported fruit and vegetable intake was not significantly different at baseline and follow up. A possible explanation may be the study juices may have replaced consumption of commercial fruit juices that could have been fortified with high levels of ascorbic acid. The reported reduction in serum vitamin C parameters is not clinically significant as mean levels remained in the clinically normal range (11-114 micromoles/L). Although under-researched in humans, some pre-clinical evidence indicates that high-doses of flavonoids may inhibit ascorbate absorption (40), which may explain the reduction in serum vitamin C. Alternatively, the reagents that were utilized to prepare the serum prior to Vitamin C analysis may have degraded over the study period.

The study limitations include a relatively small sample size and short intervention length (12 weeks). However, preclinical studies indicate that flavonoids from berry fruits may require only several weeks to accumulate in brain regions associated with cognition (41). Despite this, a short time frame limits our ability for observations regarding changes in dementia progression. Given the moderate to large effect sizes found in this study, a longer duration of follow up with larger numbers would be the next progression in research. Another limitation of the study relates to the generally better cognitive and physical ability of the intervention group, which is evident despite randomisation. Interestingly, the measure of effect for improvements on cognitive tasks in the intervention group relative to the control was the same, regardless of the cognitive ability of participants. At the baseline and 12 week visits, a standardised breakfast meal was provided, however at the 6 week visit participants arrived at the clinic facility in a non-fasted state, after having consumed their usual breakfast at home (as a fasting blood sample was not collected). If there was a large difference in the nutritional composition of their usual breakfast to the standardised breakfast (e.g. a significantly greater intake of caffeine), this may have either positively or negatively influenced cognitive performance at this time point. However, this effect was minimised by the participants consuming their breakfast at 6 and 12 weeks ad-libitum, and as the improvements in cognitive performance seen at 6 and 12 weeks are not

significantly different, a difference in breakfast is unlikely to be of great importance. Lastly, while it is likely the bioactivity of the cherry juice relates to its high anthocyanin content, the potential bioactive effects of other polyphenols cannot be excluded.

Regardless of their exact mechanisms, the potential of flavonoids to improve cognitive outcomes for older adults with Alzheimer's type dementia cannot be underestimated and a notable strength of this study is the very low possibility of any harm to this vulnerable group. Further research is required to improve the knowledge base to inform dietary recommendations for this patient group, as an adjunct to traditional dementia treatment. For older adults living with reduced cognitive capacity, the inclusion of an anthocyanin-rich beverage, in addition to or in replacement of another processed fruit juice, may be a practical way to improve their total flavonoid consumption, which has been shown to provide positive outcomes for prevention of cognitive decline.

7.4.1 Conclusion

The findings of this study suggest that regular anthocyanin-rich cherry juice consumption over 12 weeks can improve cognition in older adults with dementia and provides a basis for more comprehensive human trials to study the potential of cherry flavonoids to influence neuro-cognitive health.

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8

8.1 Overview of core findings

The research presented in this thesis utilised various methodologies to address the central research questions surrounding the measurement of dietary flavonoid intake (chapters 2, 3, 4) and the impact of dietary flavonoid supplementation on cognitive and physical outcomes (chapter 5, 6, 7).

In order to associate the impact of dietary flavonoids on health outcomes, accurate measures of dietary flavonoid intake are fundamental. The gaps in knowledge surrounding how dietary flavonoid intake is measured, presented in the introduction (chapter 1) to this thesis, indicated the need for improved methods for measuring dietary flavonoid consumption. A systematic evaluation of the various methods employed by current literature to measure flavonoid intake, and their associated limitations were highlighted in a literature review (chapter 2). The review emphasised the reliance on studies that utilise flawed or limited dietary assessment methods to measure dietary flavonoid intake. Additionally, the review established that despite several studies outlining the development and validation of biomarkers of flavonoid intake, few studies actually utilised biomarkers as an objective measure of dietary flavonoid intake.

In response to the limitations to the current methods of measuring dietary flavonoid intake, novel methods of measuring flavonoid intake were developed. Chapter 3 of this thesis identified that the measurement of dietary flavonoids in older Australians was inadequate and consequently performed secondary data analysis of detailed dietary information to describe flavonoid intake in this population (chapter 3.1). Consequently, a tool for the estimation of dietary flavonoid intake in this cohort was developed based on these findings (chapter 3.2). The tool was adequately validated against food records and assessed for reliability in a group of older Australians, and proved to be a relatively useful tool for application to measure flavonoid intake in future research in older Australians. However, the tool needs to be refined further to reduce participant burden (perhaps to shorten in length) and to assess for its usefulness beyond that of currently applied dietary assessment tools.

A sensitive and specific GC-MS method was developed (chapter 4) with the potential to measure around 115 flavonoid-related biomarkers in biological samples. The potential application of this method in future research is vast, especially for exploratory metabolomics-related studies, in which this GC/MS method could be applied to determine the most suitable biomarker related to flavonoid intake depending on the research objectives. However, the usefulness of this biomarker assessment method for determining total dietary flavonoid intake needs to be addressed. It is unlikely that a metabolite profiling approach for the measurement of dietary flavonoid intake is a straightforward technique, and therefore its application relative to dietary assessment methods must be investigated.

Overall, the dietary flavonoid intake measurement strategies developed by this thesis improved upon current methods (1, 2) of measuring flavonoid intake, and contributed novel tools with potential for application in future research studies.

In order to inform the development of interventions to assess the impact of dietary flavonoid intake on cognitive and physical outcomes, a literature review of relevant current findings was required. A literature review (chapter 5) identified a paucity of research regarding food-based anthocyanin consumption and cognitive outcomes. However, the preliminary findings indicated positive cognitive outcomes associated with anthocyanin-rich food consumption. Various methodological limitations were highlighted from the acute and longer-term trials included in the review, including issues surrounding dosages and cognitive tools employed. The findings of the review were utilised to develop acute (chapter 6) and longer-term (chapter 7) intervention studies which assessed the impact of a flavonoid and anthocyanin-rich cherry juice on cognitive and physical outcomes.

A pilot cross-over study assessed the acute effects on cognitive function, blood pressure and plasma biomarkers associated with consumption of a 300ml cherry juice, provided either as a single quantity or as 3x100ml doses administered over 2h. The methodology used in this acute pilot study contributed to an improved study design for flavonoid-based trials of cognition, with the results indicating that the acute impact of anthocyanin-rich cherry juice consumption on cognition was not supported.

However, a potential dose-timing administration effect may influence acute blood pressure outcomes. The application of the GC/MS method developed in chapter 4, measured eight related biomarkers of anthocyanin-intake, confirming the metabolism and uptake of cherry-related anthocyanins in this study.

The final study of this thesis comprised a longer-term intervention, which was conducted to assess whether daily consumption of anthocyanin-rich cherry juice changed cognitive function in older adults with dementia, with blood pressure and anti-inflammatory effects examined as secondary outcomes. The results of this study indicated that anthocyanin-rich cherry juice consumption improved cognitive performance and significantly reduced blood pressure, but did not impact markers of inflammation in this group. This study contributed significant findings to the limited human clinical trial literature regarding flavonoid supplementation and cognitive outcomes, in a group of individuals who may be most likely to benefit from improvements to cognitive performance. This study provided the first evidence that regular consumption of a feasible serving of flavonoid-rich food may have beneficial consequences in older adults with Alzheimer's type dementia, and these findings warrant further investigation.

Overall, the findings of these studies support the hypothesis that dietary flavonoids, and especially anthocyanin-rich food, is beneficial for cognitive and physical outcomes (3).

8.2 Strengths and limitations

Each study comprising this thesis had its own set of methodological strengths and limitations, which have been addressed in the preceding chapters. Consequently, some general strengths and limitations are considered here.

This thesis has highlighted that there are considerable limitations relating to the accurate measurement of dietary flavonoid intake, which hinders the ability for convincing associations between flavonoid intake and health outcomes to be drawn (2). Study 1, 2 and 3 of this thesis addressed significant gaps in knowledge surrounding

the estimation of dietary flavonoid intake, and the tools developed within these studies can be usefully applied in future research. However, the dietary assessment method (FFQ) developed and validated to measure dietary flavonoid intake is specific for the older Australian population, and thus may have limited generalisability outside of this setting. Additionally, the validity of this tool could be further strengthened by evaluating its association with flavonoid-related biomarkers through the application of the method of triads (4, 5). This work is planned by our research team, in conjunction with collaborators at the QLD Government's Department of Agriculture and Fisheries and the University of Queensland, Australia, but is outside the scope of this thesis.

Our laboratory work has shown that the GC/MS method is able to determine flavonoid-related biomarkers, which is suitable in exploratory studies to confirm the metabolism and uptake of flavonoids. However, its ability to measure biomarkers as a measure of total dietary flavonoid intake required further validation. While similar methods have shown to be useful indicators of flavonoid intake associated with pure flavonoid supplementation (6), the application of the GC/MS method to measure dietary flavonoid intake in population-based research needs further investigation.

This thesis has additionally emphasised the considerable limitations in research that investigates the cognitive and physical benefits of flavonoid consumption, and specifically foods that are rich in anthocyanins. Study 4, 5 and 6 of this thesis addressed significant gaps in knowledge relating to the cognitive and physical benefits of anthocyanin-rich food supplementation. Theses studies contribute to evidence for the dose-timing administration of flavonoid-rich food supplementation in acute trials, the cognitive benefits associated with anthocyanins in a little researched fruit variety (cherries) and the first investigations of the neuro-cognitive benefits of anthocyanin-rich food supplementation in older adults with dementia. While the contributions provided by this thesis are promising, the relatively small sample sizes of the intervention studies limit the interpretation of the results. The positive results indicated in both the acute and longer-term supplementation trials indicate that further research on a larger scale is required to confirm the findings.

8.3 Future directions

While this thesis has contributed novel investigations to the literature regarding the measurement of dietary flavonoid intake and the impact of flavonoid intake on cognitive and physical functioning, it also highlights the need for further research. Based on the findings of this thesis, the following recommendations are made for future research.

Complete food composition databases for flavonoids and appropriate dietary assessment methods are essential for any flavonoid-related research. Future investigations attempting to measure dietary flavonoid intake should apply validated flavonoid specific dietary assessment methods to ensure greater precision when measuring total flavonoid intake and the intake of specific subclasses. Ideally, future estimates of flavonoid intake will consider the growth, processing and preparation conditions on the flavonoid content of foods. While investigators are responsible for selecting appropriate methods for their studies, this step also heavily involves the improvement of flavonoid food composition databases. Analytical studies are needed to fill existing gaps on commonly eaten flavonoid rich foods, and to ensure that all appropriate foods, including values for their cooked and processed alternatives, are included in flavonoid databases. The differences between flavonoid intake estimates associated with applying different flavonoid food composition databases should also be assessed.

Identification of suitable biomarkers of flavonoid intake is imperative for an objective measurement of total flavonoid intake, and/or in the intake of flavonoid subclasses. The lack of validated biomarkers of total flavonoid intake has been highlighted as a significant problem, and has been attributed to inter-individual variation in the metabolism of flavonoids (2). Validating estimates of flavonoid intake with a relevant biomarker where possible should be considered a priority for future research. However, the limitations of biomarker assessment methods should be considered, and a burden/benefit analysis of choosing this method over others (e.g. dietary assessment methods) should be a priority for studies if crucial advances are not made in the near future.

The potential therapeutic utility of flavonoid-rich food supplementation in dementia has been highlighted (7). However, further elucidation of the practicality of anthocyanin-rich food supplementation in the 'free-living' environment is needed, especially for older adults with dementia for whom nutritional changes may be difficult to sustain. Previous research has speculated that the effects of specific fruit and vegetable consumption on cognitive outcomes in a general population are likely to be subtle (8). The clinical relevance of improvements in cognitive function, or the potential for flavonoids to mediate dementia disease progression, in older adults after anthocyanin-rich food supplementation needs to be further investigated. The differences in outcomes for supplement and food based interventions needs to be elucidated. While the use of pure supplements are useful when considering flavonoid bioavailability and mechanisms of action, future research needs to consider feasible dietary interventions in order to generalise results and inform dietary messages.

As our knowledge of flavonoid–disease relationships becomes clearer, it may soon be possible to specify recommended intake levels. Interestingly, recommendations associated with phytochemicals are beginning to be included in widely disseminated dietary guidelines (9), in addition to guidelines surrounding fruit and vegetable consumption. However, much additional work is needed before evidence-based recommendations will be possible and public health policy developers must be cautious when using information regarding flavonoid consumption specifically.

8.4 Summary

In conclusion, this thesis has highlighted the current limitations associated with estimating dietary flavonoid intake and developed new tools for measuring flavonoid intake. Additionally, this research has investigated the current research surrounding the cognitive and physical benefits associated with flavonoid intake and assessed the impact of anthocyanin-rich food supplementation on acute and longer-term health outcomes.

The research constituting this thesis has extended current knowledge regarding the assessment of dietary flavonoid intake and the impact of flavonoid supplementation

on cognitive and physical outcomes. The culmination of the six studies constituting this thesis contributes to the fields of nutrition, psychology, public health and basic science, with each study contributing a novel and innovative investigation. Together, these studies advance scientific knowledge regarding the most significant gaps surrounding dietary flavonoids. In a broader context, these studies ultimately contribute to a larger wealth of knowledge regarding how diet and dietary components can influence health outcomes in humans.

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9 APPENDICES

Appendices removed for copyright reasons