Understanding dietary lutein and zeaxanthin intake: an exploration of barriers to establishing an intake recommendation to support ocular health

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Abstract

Lutein and zeaxanthin (L/Z) belong to carotenoids xanthophylls and are highly concentrated in the macula of the eye. The supplemental and dietary intake of L/Z have been associated with decreased risk and severity of age-related macular degeneration (AMD). Macular L/Z concentration, assessed as macular pigment optical density (MPOD), has been investigated as a proxy marker for AMD risk. In 2017 it was proposed that L/Z should have dietary intake targets considering their protective role. This proposal evaluated L/Z against the set of nine criteria developed by Lupton and colleagues to determine the strength of evidence to support intake targets. Criterion 3 refers to known food-concentration databases. The 2017 evaluation depended on the United States of America (US) food supply. Furthermore, the evidence to support dose-response relationships (criterion 6) largely relied on supplemental rather than dietary intake research. Therefore, the rationale for this thesis was to explore whether L/Z meet these criteria in countries other than the US.

Chapter 1 is a published narrative literature review appraising the evidence on MPOD response to dietary L/Z intake. There was minimal evidence of a dose-response relationship between dietary L/Z intake and MPOD. A large gap was that habitual dietary L/Z intake was not quantitatively monitored during intervention studies. Studies that did attempt measuring L/Z habitual intake used non-validated dietary intake tools.

Two additional gaps were identified related to determining the relationship between habitual dietary L/Z and MPOD. Firstly, the possible impact of blue light exposure from electronic device on MPOD status. Secondly, the paucity of data on food L/Z concentrations in food supplies (criterion 3), outside the USA (e.g. Australia).

These gaps are barriers to the valid measurement of habitual L/Z intake and relationships with MPOD. Therefore, the thesis aim was to determine how habitual dietary L/Z intake can best be validly and quantitatively measured.

Four studies were conducted to address this aim. Chapter 2 describes the development and validation process of two tools to quantitively monitor habitual dietary L/Z intake in healthy adults. Two screeners, with a recall timeframe of one and four weeks respectively were developed. L/Z intake reported from each screener was compared against multiple 24-hour diet recalls via Bland-Altman plot analysis to determine validity. Both screeners were significantly correlated (Spearman's rank order, $p<0.001$) but returned poor validity compared with the 24-hour diet recalls (mean difference >0.3 mg/day). This indicated that participants were unable to report comparable L/Z intake between the tools; baby spinach contributed notably to discrepancies.

Chapter 3 describes the development and validation process of the Electronic Device Use Questionnaire (EDUQ). Healthy adults reported daily hours of device use using the EDUQ and multiple 24-hour diaries. EDUQ and diaries results were compared via Bland-Altman plot analysis; returning poor validity, indicating that participants were unable to report comparable device use. Chapter 4 describes a cross-sectional study investigating whether MPOD was predicted by sex, age, estimation of electronic device use and dietary L/Z intake using the tools developed in this thesis. MPOD was not predicted by these variables in the 96 healthy Australian adults studied. Future research with more valid measurement tools should investigate this relationship further.

The food composition database in Australia reports only 26 food entries for L and none for Z. Analysis methods were not available for review. Chapter 5 describes the investigation of 12 extraction method variations on five Australian foods selected for known high L/Z concentration based on the US database. In this thesis, extraction refers to the isolation of L/Z from the food of interest for analysis of optimal recovery and measure of concentration per gram of fresh food. One variation was most optimal based on five foods for L, and four foods for Z. The L/Z concentration measured in these foods were notably higher or lower than that that reported in existing Australian and US composition databases.

Based on the work performed in this thesis, a dietary target for L/Z cannot yet be set with confidence, because the evidence available does not meet the nine criteria required in the framework proposed by Lupton and colleagues to determine dietary target values. The L/Z screener was unable to capture valid quantitative habitual dietary L/Z intake. A purposely developed questionnaire was not able to validly capture usual blue light exposure from electronic device to determine a relationship between electronic device use and MPOD. The purposely developed dietary L/Z screener, found to be invalid, indicated significant correlation between tools and simultaneous poor agreement on Bland-Altman analysis. This outcome suggests that results solely reliant on correlation statistics from prior research investigating the relationship between dietary L/Z and MPOD, or in the condition of AMD, should be interpreted with caution. Larger local L/Z food composition databases and valid tools for improved participant reporting of L/Z are needed to determine habitual L/Z intake and accurate relationship with MPOD.

Declaration by author

This thesis *is composed of my original work, and contains* no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, financial support and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my higher degree by research candidature and does not include a substantial part of work that has been submitted *to qualify for the award of any* other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications included in this thesis

The following publication has been incorporated as section 1.3.2 to 1.3.6 in Chapter 1.

Fitzpatrick N, Chachay V, Bowtell J, Jackman S, Capra S, Shore A, et al. An appraisal of trials investigating the effects on macular pigment optical density of lutein and zeaxanthin dietary interventions: a narrative review. Nutr Rev. 2022;80(3):513-24. doi:10.1093/nutrit/nuab038

Contributor	Statement of Contribution	%
Naomi Fitzpatrick	Initial conception	60
	Writing of text	65
	Proof-reading	60
	Study selection	70
	Quality appraisal	70
	Data extraction	100
	Preparation of tables and figures	70
	Synthesis of findings	70
Veronique Chachay	Initial conception	10
	Writing of text	10
	Proof-reading	10
	Supervision, guidance	20
	Study selection	5
	Quality appraisal	5
	Preparation of tables and figures	5
	Synthesis of findings	5
Joanna Bowtell	Initial conception	10
	Writing of text	5
	Proof-reading	5
	Supervision, guidance	20
	Study selection	5
	Quality appraisal	5
	Preparation of tables and figures	5
	Synthesis of findings	5
Sarah Jackman	Initial conception	5
	Writing of text	5
	Proof-reading	5

	Supervision, guidance	15
	Study selection	5
	Quality appraisal	5
	Preparation of tables and figures	5
	Synthesis of findings	5
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	Proof-reading	10
	Supervision, guidance	15
	Study selection	5
	Quality appraisal	5
	Preparation of tables and figures	5
	Synthesis of findings	5
Angela Shore	Initial conception	5
	Writing of text	5
	Proof-reading	5
	Supervision, guidance	15
	Study selection	5
	Quality appraisal	5
	Preparation of tables and figures	5
	Synthesis of findings	5
David Briskey	Initial conception	5
	Writing of text	5
	Proof-reading	5
	Supervision, guidance	15
	Study selection	5
	Quality appraisal	5
	Preparation of tables and figures	5
	Synthesis of findings	5

The following publication has been incorporated as section 2.2 to 2.7 in Chapter 2.

N. K. Fitzpatrick, S. Capra, A. Shore, D. Briskey, S. Jackman, J. Bowtell, Chachay V. Newly developed dietary assessment tools for lutein and zeaxanthin are correlated with 24-hour diet recalls, but are not a valid measure of intake in Australian and United Kingdom adults. Nutrition Research. 2024;122:68-79. doi: 10.1016/j.nutres.2023.12.010

Contributor	Statement of Contribution	%
Naomi Fitzpatrick	Initial conception	60
	Writing of text	60
	Proof-reading	60
	Data analysis	60
	Recruitment	80
	Data collection	100
	Preparation of tables and figures	70
Veronique Chachay	Initial conception	10
	Writing of text	10
	Proof-reading	10
	Supervision, guidance	20
	Data analysis	10
	Recruitment	10
	Data collection	0
	Preparation of tables and figures	5
Joanna Bowtell	Initial conception	5
	Writing of text	5
	Proof-reading	5
	Supervision, guidance	15
	Data analysis	5
	Recruitment	10
	Data collection	0
	Preparation of tables and figures	5
Sarah Jackman	Initial conception	5
	Writing of text	5
	Proof-reading	5
	Supervision, guidance	15

	Data analysis	5
	Recruitment	0
	Data collection	0
	Preparation of tables and figures	5
Sandra Capra	Initial conception	10
	Writing of text	10
	Proof-reading	10
	Supervision, guidance	20
	Data analysis	10
	Recruitment	0
	Data collection	0
	Preparation of tables and figures	5
Angela Shore	Initial conception	5
	Writing of text	5
	Proof-reading	5
	Supervision, guidance	15
	Data analysis	5
	Recruitment	0
	Data collection	0
	Preparation of tables and figures	5
David Briskey	Initial conception	5
	Writing of text	5
	Proof-reading	5
	Supervision, guidance	15
	Data analysis	5
	Recruitment	0
	Data collection	0
	Preparation of tables and figures	5

The following publication has been included as section 3.2 to 3.7 in Chapter 3.

N. K. Fitzpatrick, V. Chachay, S. Capra, D. Briskey, S. Jackman, A. Shore, Bowtell J. Assessing electronic device use behaviours in healthy adults: development and evaluation of a novel tool. BMC Public Health. 2024;24(1):186. doi: 10.1186/s12889-024-17637-4

Contributor	Statement of Contribution	%
Naomi Fitzpatrick	Initial conception	60
	Writing of text	70
	Proof-reading	60
	Data analysis	65
	Recruitment	80
	Data collection	100
	Preparation of tables and figures	70
Veronique Chachay	Initial conception	10
	Writing of text	5
	Proof-reading	5
	Supervision, guidance	20
	Data analysis	5
	Recruitment	10
	Data collection	0
	Preparation of tables and figures	5
Joanna Bowtell	Initial conception	10
	Writing of text	5
	Proof-reading	10
	Supervision, guidance	20
	Data analysis	10
	Recruitment	10
	Data collection	0
	Preparation of tables and figures	5
Sarah Jackman	Initial conception	5
	Writing of text	5
	Proof-reading	5
	Supervision, guidance	15
	Data analysis	5

	Recruitment	0
	Data collection	0
	Preparation of tables and figures	5
Sandra Capra	Initial conception	5
	Writing of text	5
	Proof-reading	5
	Supervision, guidance	15
	Data analysis	5
	Recruitment	0
	Data collection	0
	Preparation of tables and figures	5
Angela Shore	Initial conception	5
	Writing of text	5
	Proof-reading	10
	Supervision, guidance	15
	Data analysis	5
	Recruitment	0
	Data collection	0
	Preparation of tables and figures	5
David Briskey	Initial conception	5
	Writing of text	5
	Proof-reading	5
	Supervision, guidance	15
	Data analysis	5
	Recruitment	0
	Data collection	0
	Preparation of tables and figures	5

The following publication has been included as section 5.3 to 5.7 in Chapter 5.

N. K. Fitzpatrick, V. Chachay, A. Shore, S. Jackman, S. Capra, J. Bowtell, D. Briskey. Building food composition tables: extraction methods to measure lutein and zeaxanthin concentrations in select Australian foods. International Journal of Food Science & Technology. 2024. doi: 10.111/ijfs.16938

Contributor	Statement of Contribution	%
Naomi Fitzpatrick	Initial conception	60
	Writing of text	60
	Proof-reading	60
	Data analysis	65
	Recruitment	80
	Data collection	95
	Preparation of tables and figures	70
Veronique Chachay	Initial conception	10
	Writing of text	10
	Proof-reading	10
	Supervision, guidance	20
	Data analysis	5
	Data collection	0
	Preparation of tables and figures	5
Joanna Bowtell	Initial conception	5
	Writing of text	5
	Proof-reading	5
	Supervision, guidance	15
	Data analysis	5
	Data collection	0
	Preparation of tables and figures	5
Sarah Jackman	Initial conception	5
	Writing of text	5
	Proof-reading	5
	Supervision, guidance	15
	Data analysis	5
	Data collection	0
	Preparation of tables and figures	5

Sandra Capra	Initial conception	5
	Writing of text	5
	Proof-reading	5
	Supervision, guidance	15
	Data analysis	5
	Data collection	0
	Preparation of tables and figures	5
Angela Shore	Initial conception	5
	Writing of text	5
	Proof-reading	5
	Supervision, guidance	15
	Data analysis	5
	Data collection	0
	Preparation of tables and figures	5
David Briskey	Initial conception	10
	Writing of text	10
	Proof-reading	10
	Supervision, guidance	20
	Data analysis	10
	Data collection	5
	Preparation of tables and figures	5

Other publications during candidature

Conference abstracts:

Fitzpatrick, N, Chachay, V, Capra, S, Bowtell, J, Shore, A, Jackman, S, Briskey, D. Can a lutein and zeaxanthin intake screener provide useful data? Preliminary results of a validation study [ABSTRACT]. Brain And Ocular Nutrition Conference 2022, Lecture Abstracts 27-29 July 2022, Downing College, Cambridge University, UK. J Alzheimers Dis. 2022;88(s1):S1-S26. doi:10.3233/JAD-229008

Fitzpatrick, N, Chachay, V, Bowtell, J, Jackman, S, Capra, S, Shore, A, Briskey, D. Considerations for developing an Australian lutein and zeaxanthin food composition database [ABSTRACT]. 2nd Virtual International Conference on Carotenoids 2022;22. Available from:https://d2r0txsugik6oi.cloudfront.net/neon/resource/carotenoidsociety/files/VICC%202022%20Complete%20Program%20and%20Abstracts%20033022.pdf

Fitzpatrick, N, Chachay, V, Bowtell, J, Jackman, S, Capra, S, Shore, A, Briskey, D. A protocol to validate two food frequency questionnaires developed to estimate lutein and zeaxanthin dietary intake [ABSTRACT]. 1st Virtual International Conference on Carotenoids 2021;39. Available from: https://www.carotenoidsociety.org/wp-content/uploads/2022/04/Program-and-Abstract-Book-FINAL-061121-V7_0.pdf

The University of Queensland, Three Minute Thesis (3MT), July 2020, How many do you do? (School of Human Movement and Nutrition Sciences (HMNS) 3MT School Final - Winner, Faculty of Health and Behavioural Sciences (HaBS) [VIDEO] 3MT Faculty Final - Participant. Available from: https://vimeo.com/436672132

Contributions by others to the thesis

Research Honours student (Schumack, B.) assisted with data analysis of 24-hour diet recalls and bloods for the study in Chapter 4.

Statement of parts of the thesis submitted to qualify for the award of another degree

No works submitted towards another degree have been included in this thesis.

Research involving human or animal subjects

Ethical approvals obtained in this thesis are summarised in the table below and a copy of the ethics approval letter is included in Appendix A

Study Title **(Chapter)**	Approving ethics committee **(Reference number)**	Date of approval **(Appendix)**
Validation of two lutein and zeaxanthin intake questionnaires, and an electronic device use questionnaire **(Chapter 2, Chapter 3)**	University of Queensland Health and Behavioural Sciences, Low and Negligible Risk Ethics Sub-Committee **(2020001764)**	17 August 2020 **(Appendix A-1)** Amendment 7 July 2021 **(Appendix A-2)**
Investigating Associations between chronic electronic device blue light exposure, dietary xanthophylls intake and macular pigment density in humans **(Chapter 4)**	University of Queensland Human Research Ethics Committee A **(2019002736)**	28 February 2020 **(Appendix A-3)** Amendment 7 September 2020 **(Appendix A-4)**

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Keywords

Lutein, zeaxanthin, xanthophyll, dietary intake, macular pigment, macular pigment optical density, age-related macular degeneration, screen time, food analysis, validation studies

Australian and New Zealand Standard Research Classifications (ANZSRC)

ANZSRC code: 111199, Nutrition and Dietetics not elsewhere classified, 60%

ANZSRC code: 111399, Ophthalmology and Optometry not elsewhere classified 30%

ANZSRC code: 090803, Food Nutritional Balance, 10%

Fields of Research (FoR) classification

FoR code: 1111, Nutrition and Dietetics, 60%

FoR code: 1113, Other Biological Sciences, 30%

FoR code: 0908, Food Sciences, 10%

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List of abbreviations used in thesis

ANDQCC	Academy of Nutrition and Dietetics Quality Criteria Checklist
AMD	Age-related macular degeneration
ARPE-19	Adult retinal pigment epithelial cell line - 19
BIA	Bioelectrical impedance
BL	Blue light
BMI	Body mass index
COV	Coefficient of variation
CWS	Combined weekly screeners
DEXA	Dual-Energy X-Ray Absorptiometry
DST	Dietary screening tool
eBMR	Estimated basal metabolic rate
ED	Electronic device
EDUQ	Electronic Device Use Questionnaire
FSANZ	Food Standards Australia New Zealand
FCT	Food composition tables
FFQ	Food frequency questionnaire
GSTP1	Glutathione S-transferase P1
HFP	Heterochromatic flicker photometry
HPLC	High performance liquid chromatography
L	Lutein
L/Z	Lutein and zeaxanthin
MPOD	Macular pigment optical density
MZ	Meso-zeaxanthin
MS	Monthly screener
ODU	Optical density units
RCT	Randomised controlled trial
rEI	Reported energy intake
RPE	Retinal pigment epithelium
SD	Standard deviation
SEM	Standard error of the mean
StARD3	Steroidogenic acute regulatory domain 3
SDT	Suggested Dietary Target
UK	United Kingdom
USDA	United States Department of Agriculture

US	United States of America
UQ	University of Queensland
UQRDM	University of Queensland Research Data Management
WS	Weekly screener
Z	Zeaxanthin
3MT	Three Minute Thesis
24DR	24-hour diet recall
24DUD	24-hour device use diary

Chapter 1 Background

This chapter provides the rationale, literary background and aims of this research thesis "Understanding dietary lutein and zeaxanthin intake: an exploration of barriers to establishing an intake recommendation". The remaining chapters include a review of literature, the methodologies used, and results of the four research studies conducted as part of this thesis, an overall discussion of the thesis, and future directions for research.

1.1 Thesis rationale

The research proposal for this thesis was in response to a paper by Ranard et al.[1] published in 2017. This paper proposed two carotenoids lutein and zeaxanthin (L/Z) should be included in the nutrients that have recommendations for dietary intake values. The context of the proposal by Ranard et al.[1] was reliant on several United States of America (US) specific resources. This research thesis looked to explore the strength of the proposal by Ranard et al.[1] and determine whether the proposal remains feasible in other Western countries.

The proposal by Ranard et al.[1] was facilitated by the novel work of Lupton and colleagues in 2014.[2] In 2014, Lupton et al.[2] developed a 9 point criteria to be used for determining if a non-essential nutrient with biological activity, or bioactive, should be considered for establishment of a recommended dietary intake value. Required intake of essential nutrients have been developed through a deficiency-repletion model; that is, the identification of biochemical markers and symptoms that arise when intake is inadequate. [3] Intake recommendations of essential nutrients are to ensure nutritional adequacy and, in some cases, considers health optimisation or chronic disease prevention. Lupton et al.[2] proposed that many bioactive nutrients have established relationships to health optimisation and risk of chronic disease. A deficiency model is not applicable to many bioactive nutrients, **however** a recommendation for daily intake set within the context of health optimisation and chronic disease prevention may be of benefit. [4-6] An example of a nutrient that has recommendations for daily intake, known as a Suggested Dietary Target (SDT) in Australia, is dietary fibre. [7] Lupton et al.[2] argues that establishment of intake recommendation for a relevant bioactive would provide benefit to research and population outcomes by firstly, encouraging recognition of importance, which would encourage more thoughtful evaluation and determination of evidence strength statements; secondly, greater government, university, and private investment into related research; thirdly, greater population interest in potential health benefits of the bioactive nutrient; fourth, greater inclusion of the bioactive nutrient into standard assessment of dietary and nutrient intake, such as national nutrition

surveys, which may improve understanding of relationship to health; fifth, a standardisation of research methods to improve comparability between research outcomes; sixth, positive messaging accessible to population more likely to be scientifically supported; seventh, a reduction in potentially misleading information shared to populations regarding the bioactive nutrient. The 9 criteria were then developed to provide a structured screening process to assess the breadth and strength of evidence associated with a bioactive nutrient (Figure 1-1).

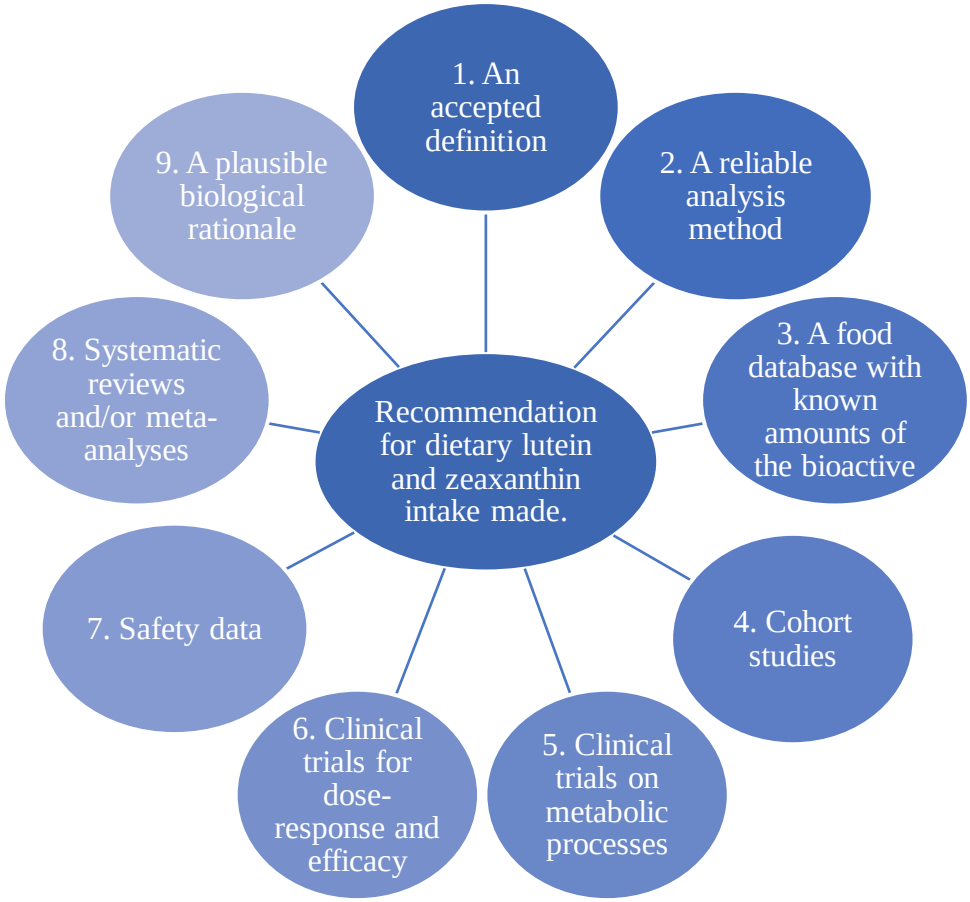

Figure 1-1 A research framework for determining recommended dietary intake for lutein and zeaxanthin, adapted from Lupton et al.[2]

Lutein (L) and zeaxanthin (Z) are two carotenoids (non-vitamin A forming) that are highly concentrated in the macula of the eye. These two polar carotenoids are isomers of one another and are categorised within the carotenoid group as xanthophylls due to their hydroxyl group. [8, 9] Lutein and Z may reduce risk of ocular conditions such as age-related macular degeneration (AMD). [10] The global prevalence of AMD has been projected to increase from 196 million in 2020 to 288 million in 2040. [11] Lutein and Z are bioactive nutrients with a plausible biological rationale for health optimisation and chronic disease prevention. Therefore, investigation of whether an evidenced-based dietary target value can be set may play a key role in preventative public health interventions to reduce the prevalence of conditions such as AMD. Ranard et al.[1] proposed that an intake recommendation should be considered for L/Z to optimise ocular health and chronic disease

prevention. Ranard et al.[1] assessed L/Z in 2017 using the 9-point criteria (see Figure 1-1) and indicated that, in the context of the US, L alone, or in combination with Z, met the 9-point criteria. When assessing criterion 3 in this proposal evidence was US-specific. Food databases may differ between countries, and these criteria may impact outcomes of the subsequent five criteria. The rationale for this thesis was to utilise the 9-point criteria as a research framework in a context outside of the US, in particular in Australia and the United Kingdom (UK). These criteria were used to identify strengths, limitations, and gaps in the evidence base more broadly and in Australia and the UK. If L/Z are to have intake recommendation, such as an SDT in Australia, a logical starting point is determining how much appears to be protective against relevant chronic diseases such as age-related macular degeneration (AMD) or optimises surrogate markers of disease risk such as macular pigment optical density (MPOD). In agreement with research outcomes highlighted by Ranard et al. [1] populations in the highest percentile of dietary intake, upwards of 3 mg/day, appear to have reduced risk of the main chronic condition of interest with L/Z, AMD. [12, 13] It is not clear however what the dietary dose-response and efficacy is of L/Z for reducing the risk of conditions such as AMD. Ranard et al. [1] justified satisfaction with this sixth criterion due to many clinical trials successfully increasing surrogate markers such as blood L/Z, macular concentrations and slowing AMD progression when individuals were provided L/Z through supplement form. However, a supplement is not dietary intake, and thus criterion six in the context of dietary L/Z intake is unclear. The initial gap to be addressed, and then used to inform the remaining projects, was a review of the literature surrounding the dose-response and efficacy of dietary L/Z interventions. A brief reflection of the Ranard et al.[1] justification that L/Z satisfy the 9 criteria raises potential enquiries as to the translatability of the justification to settings outside the US. These enquiries include dose-response efficacy of dietary L/Z intake (criterion 6), and whether countries outside the US have a database with known amounts of L/Z (criterion 3). Therefore, the rationale of this work was to:

Determine whether L/Z meet the criteria to be considered for a recommended dietary target value in Western countries other than the US.

1.2 Thesis outline

To address this, a thesis research question was developed through an initial literature review. The outcomes of this narrative review in combination with the 9-point criteria were used to identify key evidence gaps in the literature supporting dietary L/Z intake. [2] In the context of this thesis the 9-point criteria were used as a research framework that informed the study types and aims (Figure 1-1).

The narrative literature review is the first study and section 1.3 of Chapter 1 of this thesis. This review investigated the relationship between dietary L/Z interventions and MPOD. MPOD is a measure that estimates macular L/Z concentrations. The conclusions of this literature review identified the overall direction for this thesis, measurement of dietary L/Z intake. Section 1.4 of Chapter 1 presents the thesis research question and outcomes of interest, and study types for the four original projects conducted as part of this research program.

Chapter 2 details the justification and results for the second study, a validation study of two formats of a newly developed L/Z dietary intake screener. This screener was developed to address the primary outcome identified from the narrative review in Chapter 1.3. A valid and reliable way to measure dietary L/Z intake is key to research looking to address criteria 4, 6, and 8.

Chapter 3 details the justification and results for the third study conducted; a validation study of a newly developed questionnaire designed to capture usual electronic device use behaviours. This questionnaire was developed in response to the identification of blue light (BL) exposure from electronic devices (EDs) being a potential confounding factor when attempting to investigate the relationship between MPOD and dietary L/Z intake. This questionnaire does not directly address one of the 9 criteria in Figure 1-1 but was determined to be necessary to complete the fourth study and adequately respond to criteria 4–6 and 8.

Chapter 4 details the fourth study, a cross-sectional investigation of the associations between dietary L/Z intake, electronic device use and MPOD. This study looked to apply the newly developed tools from Chapters 2 and 3 and investigate whether ED use is a confounding factor when looking to understand the relationship between dietary L/Z and MPOD. The outcomes of this study were needed to inform future research looking to address criteria 4, 6, and 9.

Chapter 5 details the fifth study conducted, that is the laboratory methods to analyse food L and Z concentrations. This project addressed criteria 2 and 3 directly and provided key perspectives on whether L/Z were able to meet the 9 criteria outside of a US context. The outcomes of criterion 3 were of particular importance due to how it relates to the quality of research addressing criteria 4 to 8.

Each of the chapters describing an original research project (Chapters 2 – 5) include the review of relevant supporting literature, rationale, methods, results, discussion, and conclusions for the

specific research questions of the related study. Chapter 6 is a discussion of the findings from all prior study findings in relation to the thesis research and overarching question. Chapter 7 discusses recommendations for future research directed at improving the evidence base to support the inclusion of L/Z to have a dietary recommendation. Chapter 8 contains the references, and Chapter 9 contains the appendices.

1.3 An appraisal of trials investigating the effects on macular pigment optical density of lutein and zeaxanthin dietary interventions: a narrative review

1.3.1 Publication details

A narrative review was conducted in study one which addressed research framework criterion 6 (Figure 1-1, page 31). This review aimed to appraise the quality and findings of studies investigating the outcomes on MPOD with a dietary L/Z intervention.

Section 1.3.2 to 1.3.6 of Chapter 1 includes the manuscript published in Nutrition Reviews (Journal Impact Factor: 6.1; Quartile 1). Numbering of tables, figures, and references are presented as part of the whole thesis and as such numbering is different to that of the published work. No other text in section 1.3.2 to 1.3.6 is different to the publication.

Fitzpatrick N, Chachay V, Bowtell J, Jackman S, Capra S, Shore A, Briskey D (2022) An appraisal of trials investigating the effects on macular pigment optical density of lutein and zeaxanthin dietary interventions: A narrative review. Nutr Rev 80 (3):513-524. doi:10.1093/nutrit/nuab038

1.3.2 Introduction

Lutein, zeaxanthin and meso-zeaxanthin (MZ) are three xanthophylls, known as the macular pigments, that accumulate in the macula. The macula is part of the retina responsible for visual detail and colour vision. Thus, macular damage, as seen in age-related macular degeneration (AMD), can result in visual impairment or loss. [10] The macular pigments may play a role in optimising vision, such as visual acuity, [14] contrast sensitivity, [15] photostress recovery, [16] glare reduction, [16] and visual processing speed. [17] Additionally, the macular pigments are proposed to maintain macular health through two main mechanisms. Firstly, the macular pigments have direct and indirect antioxidant activity as demonstrated from *in vitro* studies using adult retinal pigment epithelial cell line cultures, and animal retinas dissected post-mortem. [18-23] Secondly, the macular pigments are photosensitive molecules and absorb blue visible light (400-500 nm). [24] Blue light is high energy and can stimulate the production of damaging singlet oxygen species in other macular photosensitive molecules. [24] The absorbance range of post-mortem human macular pigment samples has been shown to be between 430 nm and 490 nm, with peak absorption at approximately 460 nm. [25] The positioning and orientation of the macular pigments within the macula cell layers allow blue light absorption before it reaches other photosensitive molecules. Thus, it has been proposed that the macular pigments reduce the production of damaging singlet oxygen species in the macula. [24]

Macular lutein and zeaxanthin (L/Z) must be acquired through dietary intake, as they are not synthesized endogenously. Meanwhile, MZ is synthesised endogenously as a product of L isomerization in the retina. [26] Despite the required acquisition of L/Z from the diet and implications in macular health, a recommended dietary intake has not yet been established. However, the status of 'bioactive compounds' has been suggested. [1] The National Institutes of Health Office of Dietary Supplements defines bioactive compounds as "Bioactive food components are constituents in foods and dietary supplements, other than those needed to meet basic nutritional needs, which are responsible for changes in health status." [27] Traditionally, dietary recommendations have been developed for bioactive compounds deemed to be essential or conditionally essential through a deficiency-repletion model, and apply to protein, vitamins and minerals. [3] Ranard et al.[1] argued that L/Z meet the nine criteria recently proposed by Lupton et al.[2] to determine if a bioactive compound has the depth of evidence relating to essentiality in health to be considered for intake recommendations. [1, 2] To date, determination of an intake recommendation has been limited by the paucity of clinical data about the effects of L/Z dietary intake (as opposed to supplemental intake) on macular concentrations and health.

The concentration of the L/Z/MZ within the macula, or macular pigment optical density (MPOD), is used as a surrogate marker of macular health. [28] MPOD can be measured through a number of methods, one of which is heterochromatic flicker photometry (HFP). [29] MPOD was identified as a potential marker of macular health in a number of cross-sectional studies. These studies observed MPOD to be significantly lower in eyes of individuals with AMD compared to healthy controls. [28, 30-32] Despite the association between lower MPOD and AMD, MPOD thresholds representing 'optimal' or 'adequate' macular health for a specific age-group have not been determined. Additionally, the magnitude of MPOD change that is clinically or functionally meaningful is unclear. The lack of clarity surrounding MPOD values may partly be due to the difficulty in comparing values obtained from the different measurement methods. [29] However, a higher MPOD is generally perceived to be associated with better macular health. [28]

L/Z/MZ supplementation studies have consistently shown to result in increased MPOD. A 2016 meta-analysis that pooled results from 20 randomised controlled trials (RCTs) investigating the effects of L/Z/MZ supplementation in adults with or without AMD found a significant increase in MPOD. [33] The pooled results from nine RCTs in populations with AMD (n = 938, 50 years of age and above) showed that supplementation with L, Z and/or MZ increased MPOD by 0.07 optical density units (ODU) compared with placebo. Additionally, the dose-response relationship in this population indicated that MPOD increased by 0.005 ODU for each additional 1 mg / day in L/Z/MZ

supplementation. [33] Comparatively, the results of eleven pooled RCTs including healthy populations (n = 826, 18 years and above) showed that supplementation increased MPOD by 0.09 ODU compared with placebo. The dose-response relationship in healthy populations indicated that MPOD increased by 0.004 ODU for each additional 1 mg / day in L/Z/MZ supplementation. [33] Furthermore, a significant negative correlation was observed between baseline MPOD values and the degree of MPOD change with supplementation ($r = -0.71$, $p < 0.001$) [33], suggesting supplementation to be more effective when baseline MPOD values are lower.

In comparison to supplementation trials, there is less clarity with regard to the effects on MPOD of increasing L/Z intake through wholefoods. Understanding the impact of dietary interventions on MPOD is of interest to inform future research for the purpose of prevention of AMD. The aim of this narrative review was therefore to critically appraise reports from interventions that investigated the effect of increased dietary L/Z intake on MPOD in adults.

1.3.3 Materials and methods

The method for this review involved a systematic search with defined inclusion and exclusion criteria, data extraction, quality appraisal of all studies, and synthesis of study findings by narrative review. [34]

Inclusion criteria were: primary research papers published in English, full text availability, an intervention arm in adults increasing dietary L/Z intake through wholefood consumption, and measurement of MPOD as an outcome. A dietary intervention was deemed ineligible when the L/Z food product was prescribed in a highly concentrated form, i.e. freeze-dried powder, or liquid concentrate. No restrictions were placed on study design or year of publication. Four databases were searched up to April 2020: Pubmed, Cochrane Library, Web of Science, Cinahl. Search terms included; “retina*” OR “retinal pigment*” OR “macula lutea” OR “macular pigment” OR “macular pigment density” OR “macular pigment optical density”) AND (“lutein” OR “zeaxanthin” OR “xanthophyll*” OR “macular xanthophylls” OR “macular pigments”) AND (“diet* intake” OR “diet therapy” OR “dietary intervention” OR “diet supplement*” OR “dietary supplement*”)). Titles and abstracts of 251 papers identified in the search were screened for eligibility. Full texts were reviewed to decide on inclusion, and references were screened for any potentially relevant articles that may have been missed through electronic search methods. The literature selection process is outlined in a flow chart (Figure 1-2 [35]) adapted from the Preferred Reporting Items for Systematic Reviews and Meta-Analyses. [35]

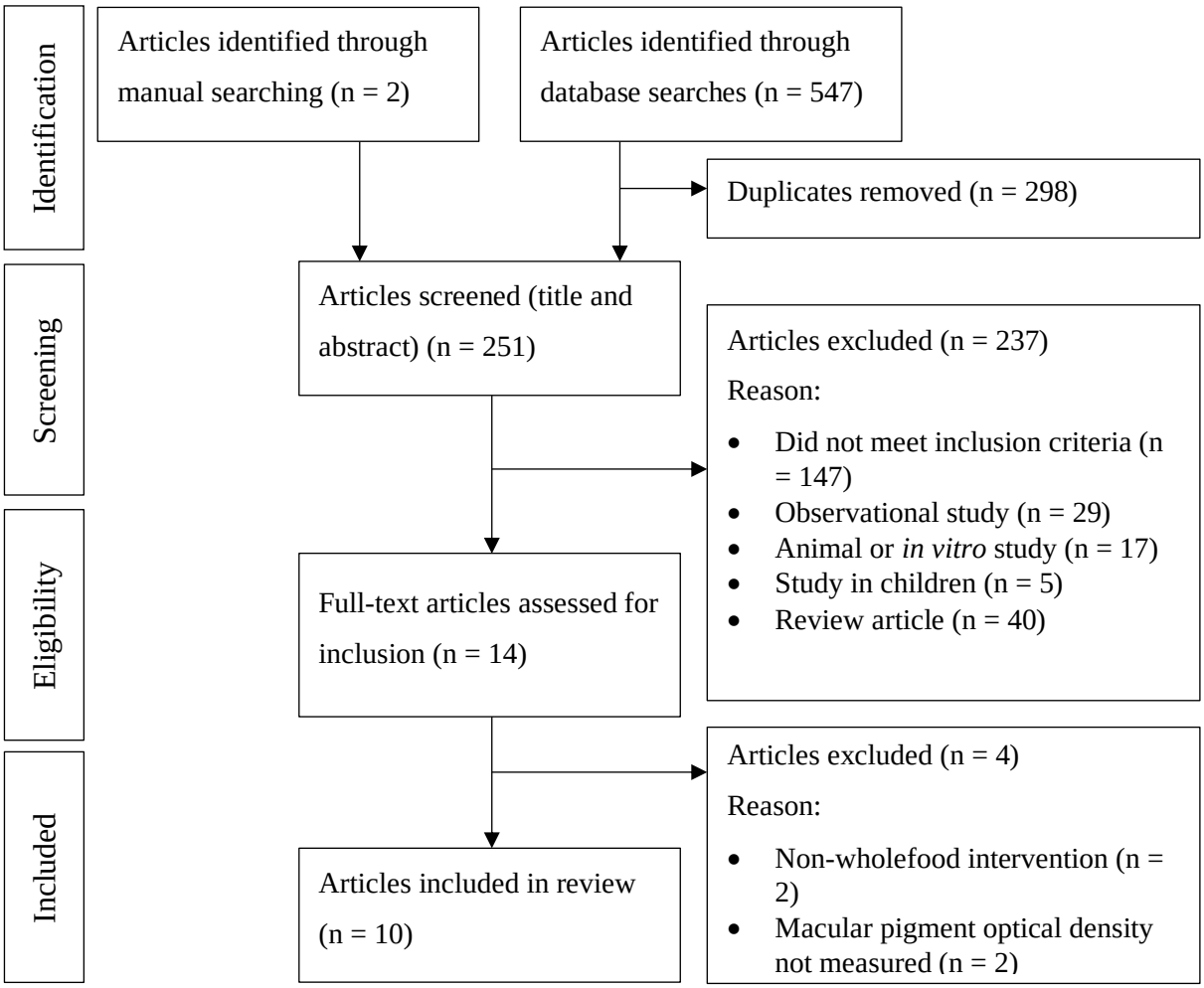

Figure 1-2 Flowchart of study selection adapted Preferred Reporting Items for Systematic Reviews and Meta-Analyses [35]

Quality appraisal of selected articles was performed using the Academy of Nutrition and Dietetics Quality Criteria Checklist (ANDQCC) for primary research. The ANDQCC contains four questions regarding the relevance of research, and ten questions relating to the validity of the research. The tool evaluates the quality of reporting of inclusion/exclusion criteria, the quality of data collection and analysis, the generalizability of results, and identifies bias in order to grade the quality of the evidence. [36] One reviewer extracted information from included studies through identification of the factors of interest including: study design, study duration, subject characteristics, dietary intervention characteristics, dietary intake measures utilized, and MPOD outcomes.

1.3.4 Results

1.3.4.1 Study characteristics

Ten studies met the inclusion criteria and were published between 1997 and 2020. Study characteristics and outcomes are summarised in Table 1-1 [37-46]. The ten studies included 613

(62% female) adults participants aged 18 to 92 years, with study sample sizes ranging from 13 to 114 participants. There were seven RCTs, [37-39, 41, 42, 44, 45] one single-blind non-randomised controlled trial, [43] one open label intervention, [46] and one cross-over study. [40] All studies measured MPOD by HFP. Specific inclusion criteria across the ten studies included AMD status, sex, age, body mass index (BMI), and habitual dietary L/Z intake. For the purpose of this review, habitual dietary intake refers to dietary L/Z intake outside of the intervention food consumption. Eight studies were conducted in healthy individuals, [37-41, 43, 45, 46] and two in individuals with early AMD. [42, 44] One study investigated exclusively female participants, [39] and three studies only included individuals 50 years or older. [37, 40, 42] Two studies included individuals with a BMI of 30 kg/m^2 or less, and one study a BMI 25 kg/m^2 or more. Lastly, only one study considered habitual dietary L/Z intake as part of the recruitment inclusion criteria. [37] Scott et al.[37] used a three-question tool to screen for intake low in L rich foods. Only participants consuming less than three serves per week of leafy vegetables, broccoli and/or eggs were included in the study. [37]

Seven studies met the criteria to receive a positive quality rating based on the ANDQCC for primary research, [37, 38, 40-42, 44, 45] and three studies a neutral rating. [39, 43, 46] One study did not provide adequate information regarding the selection and characteristics of participants. [46] One study did not clearly outline how participant group assignment occurred, and reported that mean baseline MPOD was significantly different between all three groups ($p < 0.05$). [39] Seven studies reported attrition rates, and rates ranged between 3% and 36%. [37, 38, 40-43, 46] Reasons for attrition included dislike of intervention food, or gastrointestinal discomfort. [37, 40, 42] Furthermore, poor adherence to intervention protocol resulted in data exclusion at the time of analysis in one study. [38]

All studies provided adequate detail regarding the intervention prescription and utilised an appropriate tool to measure the primary outcome of interest, MPOD. [47] However, intervention adherence was monitored only in six studies, [37-40, 43, 46] and data reported only for two studies. [37, 38] In these two studies, participants' dietary intervention adherence was greater than 90%. [37, 38] Methods to monitor adherence included diet diaries and food frequency questionnaires in four studies, [37, 38, 40, 46] return of empty food containers in two studies, [39, 40] dietitian-administered interviews in two studies, [37, 40] and supervision during food consumption by a study investigator in one study. [43] Habitual dietary intake was a secondary outcome that was assessed and reported in only four studies. [37, 40, 43, 46] Eight studies reported clear and appropriate statistical methods. [37, 38, 40-44, 46] Two of the RCTs did not report between-group analyses, and only considered change over time within group. [39, 45]

The dietary interventions involved provision of a one or two specific foods without change to the overall habitual dietary pattern, termed *prescriptive dietary intake* hereinafter. As summarised in Table 1-2, for the nine studies that reported the intervention dosage of L/Z/MZ, the median dose was 0.98 mg/day (range = 0.26–17.58 mg/day). One study reported the L/Z/MZ dosage as a combined value, [38] all other studies reported dosage of L, Z, and/or MZ individually. The frequency of consumption was daily in seven studies, [37, 38, 40-42, 44, 46] six days weekly in one study, [39] and 5 days weekly in two studies. [43, 45] The intervention food was avocado (two studies) (0.5–0.7 mg/day L/Z), [37, 38] egg (five studies) (0.26–1.88 mg/day L/Z), [39-43] goji berries (17.58 mg/day L/Z), [44] spinach (3–4.32 mg/day L), [45] or a combination of spinach and corn (11.8 mg/day L/Z) in the ten studies. [46]

Eight of the ten studies included a control group. The control intervention included isocaloric amount of potato (0 mg L),[37] isocaloric meal without avocado (0.16–0.21 mg L/Z), [38] continuation of habitual diet, [41, 44, 45] prescription of a sugar capsule (0 mg L/Z), [39] buttermilk drink (0 mg L/Z), [42] or non-xanthophyll enriched egg as control in the xanthophyll enriched egg study. [43] Xanthophyll concentration in enriched and control eggs were monitored but values not reported. [43]

1.3.4.2 Effects of dietary interventions on macular pigment optical density

Only two of the eight controlled studies reported a statistically significant increase in MPOD between the intervention and control groups, as seen in Table 1-1. [42, 44] Of these two studies, the first study reported a 16% MPOD increase after 12-months ($p < 0.05$), [42] and the second study reported a 20% MPOD increase after three months ($p = 0.007$). [44] Both of these studies were in adults with early AMD aged 50 years or above, with sample sizes greater than 100. The other five controlled trials either reported no significant differences between groups, [37, 38, 41, 43] or did not report performing between-group analyses. [39, 45] One of the two trials without a control group reported a significant MPOD increase from baseline by 14 weeks ($p < 0.05$), absolute values were not reported. [46] Across the eight controlled studies, no significant changes in MPOD in the control group were observed except in one avocado based trial. In this trial, a significant MPOD increase of 17% from baseline was reported at the halfway point of the intervention in the control group receiving potato (0 mg L). However, statistical significance was not maintained by the end of the study. [37] No changes in habitual dietary intake were reported for the control group, as monitored by dietitian-administered interviews.

1.3.4.3 Effects of dietary interventions on blood lutein and zeaxanthin concentrations

Blood concentration of L was measured in all studies, Z in nine studies, [37, 39-46] and MZ in one study [43], as seen in Table 1-2.

Only three of the eight controlled studies reported a significant increase in blood L response compared control. [38, 41, 43] Interestingly, no significant MPOD changes were observed in these three studies. A significant increase from baseline in mean blood L concentration ranging from 22% to 126% was observed within the intervention groups in nine studies. [37-43, 45, 46] A significant increase was also observed in the control groups in two studies. [37, 43] In the first study, a 15% increase from baseline was observed at six months ($p = 0.03$). [37] This control group was provided meals containing 0 mg L/Z and requested to make no other dietary changes. In the second study, a 31% increase from baseline was observed at eight weeks in the control group ($p = 0.007$). [43] This control group were provided a normal egg containing L/Z and requested to make no other dietary changes. Meanwhile, the intervention group in this study received egg enriched with L and MZ.

Three of the eight controlled studies reported significant increases in blood Z concentration compared to the control. [41, 43, 44] A significant MPOD increase was observed in only one of these three studies [44]. A significant increase from baseline in mean blood Z concentration ranging from 36% to 337% was observed in the intervention groups in six studies. [39-44] Of note, significant increase from baseline in mean blood Z concentrations was also observed in the control groups of two studies. [37, 43] In the first of the two studies, a 20% increase from baseline was observed at six months ($p = 0.004$). [37] In the second study, a 41% increase from baseline was observed at eight weeks ($p = 0.009$). [43] These two control groups were two of the three control groups that also reported significant blood L changes.

One study monitored blood MZ, and MZ was not detectable at baseline for either the control or intervention group. [43] At eight weeks, blood MZ was significantly increased compared to the control group which observed no change ($p < 0.001$). [43]

1.3.4.4 Dietary intake measurement

Habitual dietary intake was assessed and reported in only four of the ten studies, and assessed using different tools as seen in Table 1-1. [37, 40, 43, 46] Scott et al.[37] used two types of measures: a 132-item semi-quantitative food frequency questionnaire (FFQ) with a recall timeframe of 12 months, and dietitian-administered interviews. [37] The FFQ was not specifically validated to

quantify L/Z dietary intake. It was administered at baseline and the mean daily L/Z dietary intake was calculated from a food composition analysis software (Nutrition Data System for Research software (version 2016). The mean L/Z consumption for the intervention and control groups were not significantly different (3.0 ± 3.1 mg/ day and 2.8 ± 2.7 mg/day respectively). The dietitian-administered interviews were conducted monthly to monitor maintenance of dietary habits. No significant change in habitual dietary intake was identified, but details of the interview questions were not reported. [37] In the study by Vishwanathan et al.[40] a 7-day diet diary was completed once by participants during each study phase. Total L/Z intake was not quantified, but the diaries were reviewed for intake of foods known to contain 'substantial' amounts of L/Z. Whilst the criteria for 'substantial' was not defined, the intake of spinach, broccoli and corn were monitored. Intake of these three foods were reported to contribute approximately 0.3 mg/day during the study phases. [40] In the study by Kelly et al.[43], a dietary screening tool (DST) was used at baseline to infer whether habitual dietary L/Z intake was high or low. [43] The DST estimates overall dietary quality graded in three categories based on adherence to the American Dietary Guidelines. The 'at-risk' DST category has been correlated with lower serum L/Z concentration, when compared to the 'possible risk' or 'not-at-risk' categories. [48] The DST does not however quantitatively estimate L/Z intake. In the study by Hammond et al.[46], dietary intake was measured at baseline with the Health Habits and History Questionnaire, developed from the American National Health and Nutrition Examination Survey II data. [49] The Health Habits and History Questionnaire is not validated to specifically quantify L/Z dietary intake. Participants' L/Z intake was calculated from the questionnaire data using a food composition database, but values were not reported. [46] Therefore, only one of the ten studies quantified and reported baseline habitual L/Z dietary intake. [37] None of the studies quantitatively monitored and reported habitual dietary L/Z intake over the study duration.

375 Table 1-1 Study interventions and outcomes

Author (date) [study quality]	Study design	Participant characteristics	Inclusion criteria	Intervention (mg L/Z/MZ per food serve)	Mean MPOD			Blood L/Z/MZ response		Method to monitor habitual dietary intake
					baseline (ODU ± SD)	Study end (ODU ± SD)	% change from baseline	L % change from baseline	Z % change from baseline	
Treatment food: avocado										
Scott et al. (2017) [37] [+]	RCT, 26 weeks	n = 40 (52% female), ≥ 50 years	Healthy	G1: 135 g/day avocado (0.5 mg L)	G1: 0.39 ± 0.14	G1: 0.49 ± 0.14	G1: 26% **c**	G1: 26% **c**	G1: -10%	Baseline semi-quantitative, 132-item FFQ and monthly dietitian administered interviews.
				G2: potato (0 mg L)	G2: 0.38 ± 0.17	G2 0.42 ±0.15	G2: 11%	G2: 15% **b**	G2: 20% **b**	
Edwards et al. (2020) [38] [+]	RCT, 12 weeks	n = 84 (63% female), 25-45 years	Healthy, BMI ≥ 25 kg/m^2	G1: 1x 527-659 calorie meal/day with avocado (0.56-0.7 mg L/Z)	G1: 0.47 ± 0.22	G1: 0.50 ±0.21	G1: 6%	G1: 33% **b ***	G1:NR	Not monitored.
				G2: 1x 529-662 calorie meal/day no avocado (0.16-0.21 mg L/Z)	G2: 0.47 ± 0.19	G2: 0.49 ± 0.20	G2: 5%	G2: -7%	G2: NR	
Treatment food: egg										

Wenzel et al. (2006) [39] [Ø]	RCT, 12 weeks	n = 24 (100% female), 24-59 years	Healthy, BMI ≤ 30 kg/m^2	G1: 6 eggs/week (0.20 mg L, 0.13 mg Z)	G1: 0.18 ± 0.02 a	Values NR	G1: **c**	G1: 23% **b**	G1:NR **b**	Not monitored.
				G2: 6 eggs/week (0.60 mg L, 0.37 mg Z)	G2: 0.37 ± 0.06 a		G2: **b**	G2: 26%	G2: NR **b**	
				G3: 1 x sugar pill/day (0 mg L/Z)	G3: 0.29 ± 0.04 a		G3	G3: 10%	G3: NR	
Vishwanathan et al. (2009) [40] [+]	Cross-over trial, 4 week run in, 5 week intervention, 4 week break, 5 week intervention	n = 52 (60% female), ≥ 60 years	Healthy	Phase 1: 2 egg yolks/day (0.44 mg L, 0.46 mg Z)	0.49 ± 0.04 (at 0.5 ºE)	Phase 1: 0.52 ± 0.04 (at 0.5 ºE)	Phase 1: 6% (at 0.5 ºE)	Phase 1: 16% **b**	Phase 1: 36% **c**	7-day diet diary once per study phase (4 total).
				Phase 2, 4 egg yolks/day (0.96 L, 0.92 Z)		Phase 2: 0.54 ± 0.03 (at 0.5 ºE)	Phase 2 (10%) (at 0.5 ºE)	Phase 2: 24% **c**	Phase 2: 82% **c**	
Kelly et al. (2014) [41] [+]	RCT, 12 weeks	n = 97 (59% female), ≥ 18 years	Healthy, BMI ≤ 30 kg/m^2	G1: 1 non-enriched egg/day (0.17 mg L, 0.9 mg Z)	G1: 0.31 ± 0.14	G1: 0.35 ± 0.22	G1: 13%	G1: 9%	G1: 64%	Not monitored.

				G2: 1 L enriched egg yolk in buttermilk drink (0.97 mg L, 0.34 mg Z)	G2: 0.38 ± 0.12	G2: 0.32 ± 0.16	G2: -16%	G2: 78% **c ***	G2: 93%	
				G3: 1 L enriched egg/day (0.92 mg L, 0.14 mg Z)	G3: 0.32 ± 0.12	G3: 0.36 ± 0.16	G3: 13%	G3: 60% a **c ***	G3: 92%	
				G4: 1 Z enriched egg/day (0.17 mg L, 0.49 mg Z)	G4: 0.35 ± 0.14	G4: 0.36 ± 0.21	G4: 2%	G4: 14%	G4: 337% **c ***	
				G5: nil change to diet	G5: 0.34 ± 0.15	G5: 0.35 ± 0.17	G5: 3%	G5: -2%	G5: 47%	
Van der Made et al. (2016) [42] [+]	Double-blind RCT, 52 weeks	n = 101 (67% female), ≥ 50 years	Early AMD, visual acuity >0.5	G1: 1.5 L enriched egg yolk in buttermilk drink (1.38 mg L, 0.21 mg Z)	G1: 0.45 ± 0.14	G1: 0.52	G1: 16% **c ***	G1: 94% **c**	G1: NR b	Not monitored.
				G2: buttermilk drink no egg yolks (0 mg L/Z)	G2: 0.46 ± 0.16	G2: 0.48 (SD NR)	G2: 4%	G2: NR	G2: NR	

Kelly et al. (2017) [43] [Ø]	Placebo controlled trial, 8 weeks	n = 50 (38% female), 18-65 years	Healthy	G1: 1 L, Z, and MZ enriched egg/day (values NR) G2: 1 non-enriched egg/day (values NR)	G1: 0.45 ± 0.20 G2: 0.41 ± 0.17 (at 0.5 ºE)	G1: 0.41 ± 0.21 G2: 0.44 ± 0.20 (at 0.5 ºE)	G1: -9% G2: 7% (at 0.5 ºE)	G1 126% **c** * G2: 31% **b**	G1: 68% **c** G2: 41% **b** MZ not detected at baseline for G1 or G2, and detected at 0.084 µmol/L for G1 only by week 8 **c** *	Dietary Screening Tool at baseline.
Treatment food: goji berries										
Li et al. (2018) [44] [+]	RCT, 12 weeks	n = 114 (70% female), 51-92 years	Early AMD	G1: 25g/day goji berries (2.5 mg L, 15.08 mg Z) G2: nil change to diet	G1: 0.73 ± 0.21 G2: 0.72 ± 0.19	G1: 0.88 ± 0.20 G2: 0.76 ± 0.19	G1: 21% **c** * G2: 6%	G1: 2% G2: NR	G1: 248% **c** * G2: 7%	Not monitored.
Treatment food: spinach										
Kopsell et al. (2006) [45] [+]	RCT, 12 weeks	n = 30 (70% female), 21-60 years	Healthy	G1: 50 g high L variety spinach 5	G1: 0.34 ± 0.04	G1: 0.34 ± 0.04	G1: 9% **b**	G1: 49% **b**	G1: 36%	Not monitored.

				days/week (6.05 mg L)					
				G2: 50 g lower L variety spinach 5 days/week (4.2 mg L)	G2: 0.35 ± 0.04	G2: 0.35 ± 0.04	G2: 0%	G2: 28% **b**	G2: -36%
				G3: nil change to diet	G3: 0.31 ± 0.04	G3: 0.31 ± 0.04	G3: 0%	G3: 5%	G3: -11%
Treatment food: spinach and corn									
Hammond et al. (1997) [46] [Ø]	Open label intervention trial, 14 weeks	n = 10 (69% female), 30-65 years	Healthy	G1: 60 g spinach/day, 150 g corn/day (11.2 mg L, 0.6 mg Z)	Values NR		G1: b	G1: NR **b**	G1: NR	Healthy Habits and History Questionnaire at baseline

Study quality assessed by ANDQCC for primary research: (+) relevant and valid study, low risk of bias; (Ø), relevant study, moderate or unclear validity and risk of bias [36]. [a] significant difference between groups at baseline $p < 0.05$, [b] significant MPOD increase from baseline $p < 0.05$, [C] $p \leq 0.001$, ***** significant MPOD change versus control group $p < 0.05$. Abbreviations: AMD, age-related macular degeneration; BMI, body mass index; °E, degrees eccentricity from macular centre; G, group; L, lutein; MPOD, macular pigment optical density; n= number of participants; NR, not reported; ODU, optical density units; %, percentage; SD, standard deviation; Z, zeaxanthin.

1.3.5 Discussion

This narrative review aimed to critically appraise reports from interventions that investigated the effect of increased dietary L/Z intake on MPOD in adults. A varied MPOD response was observed. The reason for this variation is difficult to determine due to substantial heterogeneity between studies, and limited monitoring of habitual dietary L/Z intake. Only two of the eight controlled studies reported significant increases in MPOD in the intervention group. [42, 44] Of these two studies, only one also observed significant change in blood Z concentrations. [44] The other studies observed significant changes in blood L/Z/MZ concentrations, but without significant MPOD change. Heterogeneity in trial design and participant characteristics between studies may explain the inconsistences between study results, and inform future study design. Identified heterogeneity between the studies included the variety of prescribed intervention foods, L/Z dosage, intervention duration, and differences in participant characteristics such as age, sex, AMD status, body composition, baseline MPOD and habitual dietary L/Z intake.

1.3.5.1 Influence of participant characteristics on macular pigment optical density response

Participant habitual dietary lutein and zeaxanthin intake.

A quantitative value for habitual L/Z dietary intake was reported at baseline in only one of the ten studies, [37] and measured but not reported in two studies. [40, 46] The importance of quantitively monitoring habitual dietary L/Z intake is highlighted in the study by Scott et al.[37] The baseline intake of the intervention and control group was reported to be 3.0 ± 3.1 mg/day and 2.8 ± 2.7 mg/day respectively. [37] Following baseline, a significant MPOD increase from baseline of 17% was reported at three months in the control group. [37] This MPOD change was not maintained at six months, but serum L/Z was significantly elevated. Of note, no changes in dietary intake were reported, and intake was monitored by dietitian-administered interviews for which question details were not reported. Thus, the potential impact of change to habitual dietary intake, such as due to seasonal variation in available foods, cannot be quantitatively determined. The high baseline inter-individual variability also highlights the need for quantitative measurement of habitual L/Z dietary intake to determine whether the amount of L/Z prescribed as part of a dietary intervention is a small, moderate or large change relative to a participant's habitual intake. In the study by Scott et al.[37] the variable baseline dietary L/Z intake of the intervention group (3.0 ± 3.1 mg/day) meant the prescribed intervention of 0.5 mg/day of L was highly variable in how much it increased participants' total L/Z intake. [37] Thus, quantitative estimation of habitual L/Z intake is critical to measure over the whole study duration when considering the high inter-individual variability reported at baseline, the MPOD change observed in the control group, and lack of significant MPOD change observed between the intervention and control group. Furthermore, the lack of

continuous quantitative measurement is a substantial limiting factor when interpreting the MPOD response observed.

The importance of monitoring habitual dietary L/Z intake over the study duration is demonstrated again in the cross-over trial from Vishwanathan et al.[40] In this study, the three foods (broccoli, spinach and corn) analysed from 7-day diet diaries performed once during each study phase contributed 0.3 mg/day of L/Z in each phase. [40] The 0.3 mg/day of L/Z provided the equivalent of 33% of the phase 1 egg dosage (0.9 mg/day), and 16% of the phase 2 egg dosage (1.88 mg/day). Relative to the intervention L/Z dose prescribed, dietary L/Z intake from just three foods were measured to contribute a substantial amount of the total L/Z being consumed by participants. As a factor that may influence MPOD outcomes, measurement of total habitual L/Z intake, not just from three foods, is therefore critical to consider when interpreting the MPOD response observed.

Habitual L/Z dietary intake was not quantitively monitored over the full study duration in any of the studies. Therefore, it is unclear for the ten studies in this review whether habitual L/Z dietary intake influenced reported MPOD outcomes. The lack of habitual L/Z intake monitoring in these studies is a serious limitation and should be considered when interpreting MPOD outcomes in this review and in future research. To effectively monitor habitual dietary L/Z intake in future studies, standardisation of the dietary intake tools utilised is needed. Four of the ten studies in this review did assess habitual intake at one point throughout the study. [37, 40, 43, 46] However, each study utilised different dietary intake tools, and none of these tools had been specifically validated to monitor dietary L/Z intake. To our knowledge, there are currently no dietary intake tools specifically designed to quantitatively monitor habitual dietary L/Z intake. The development of such a tool is warranted.

Participant macular pigment optical density.

The variable MPOD response observed in the ten studies reviewed may have also been influenced by the protocol utilised to measure MPOD, HFP. HFP has been shown to have high test-retest reliability. However, HFP is a psychophysical measure as it relies on adequate participant input and understanding of the activity to complete the measure. As such, when using HFP, the effect of participant practice in measurement completion has been acknowledged as an important methodological consideration. [50] A minimum of two measurements of MPOD per session has been recommended to monitor the influence of intra-person variability and ‘practice effect’ associated with performing HFP. [47] Only four of the studies in this review clearly indicated that participants were familiarised and provided with education to understand the HFP procedure. [37,

41-43] Five of the studies reported using the mean of three or more repeated MPOD measurements at a single timepoint, [37, 39, 40, 44, 45] and one study reported measuring twice at baseline but did not clearly indicate which value was utilised. [46] Four studies did not clearly indicate that repeat measures were conducted. [38, 41-43] Thus, for these four studies whether the change in reported MPOD values is due to true change or due to the practice effect cannot be determined. In addition to the practice effect, MPOD values obtained were difficult to compare between studies due to multiple different HFP machines and protocols utilised. One study used a Maxwellian view system, [46] two studies used the QuantifEYE Macular Pigment Screener II [51], and seven studies used the Macular Densitometer [52]. These HFP machines and protocols differ in aspects such as degrees of eccentricity measured from the fovea in the macula, wavelengths of light used for measurement, accommodation of inter-individual differences in flicker thresholds, and whether an individual is looking for a flicker to appear or disappear.[53] These differences between HFP methods may result in different MPOD values measured, and is described in detail in a review of MPOD techniques by Howells et al.[53]. Future research utilising HFP would be strengthened through completion of a minimum two MPOD measures at each time point as standard practice recommends, and reporting of the within-session variability, such as by coefficient of variation or similar reliability measures. Alternatively, utilisation of objective MPOD measures in future research, such as fundus autofluorescence, would remove the influence of the practice effect. [53]

Another factor that may influence MPOD response with increased L/Z intake is participant baseline MPOD. [33] Lower baseline MPOD has been associated with a greater MPOD response to L/Z supplementation. [33] In two of the ten studies in this review, the observed absence of MPOD response was proposed to be due to the high baseline participant MPOD. [38, 40] However, this association of baseline MPOD influencing responsiveness to elevated L/Z/MZ intake does not appear as convincing in the studies within this review. Participants' mean baseline MPOD was above 0.38 ODU in three of six studies reporting statistically significant MPOD improvements from baseline, and was as high as 0.7 ODU (a study also reporting significant MPOD increase compared to the control group). [37, 42, 44] Any attempt to interpret the potential influence of baseline MPOD on responsiveness to elevated dietary L/Z intake is made more difficult by the inability to consider the influence of habitual dietary L/Z intake in this relationship. Without habitual dietary L/Z intake data, it cannot be determined whether baseline habitual intake is related to the baseline MPOD values and subsequent responses observed. Further research is needed to investigate the difference in MPOD response in participants with a baseline MPOD above or below 0.4 ODU when prescribed the same dietary L/Z intervention.

Other participant characteristics.

There was heterogeneity in the age, sex, AMD status, and body composition of participants across the ten studies. Age and sex are not generally considered to be independent determinants of MPOD status, [51, 54] while AMD has been associated with lower MPOD status. [28, 30-32] The heterogeneity in AMD status of participant groups resulted in additional difficulty when attempting to compare studies to interpret the trends in MPOD outcomes in relation to the intervention food used, L/Z dose provided, and intervention duration.

Two of the ten studies suggested that the absence of any statistically significant increase in MPOD may have occurred due to the higher body fat composition of the study population. [38, 40] This suggestion was based on the BMI being 25.0 kg/m^2 or greater in these participants. As L/Z are fat soluble nutrients they can be deposited in adipose tissue, [55] although mechanisms regulating carotenoid uptake or release from adipose tissue are not well understood. [56] Higher percentage of body fat has been previously inversely associated with MPOD. [55] However, in two of the ten studies, participants' BMI was 25.0 kg/m^2 or greater, and yet significant MPOD improvement was observed [42, 45]. Intervention group MPOD increased significantly compared to the control group in one study, [42] and compared to baseline in the other study. [45] Clearly, BMI is not an accurate measure of body fatness, and as such it is not possible to draw definitive conclusions regarding the influence of body fat percentage on MPOD response. None of the ten studies measured body fat percentage, thus future studies may benefit by including robust measurement of body composition. An additional consideration is the current lack of understanding surrounding mechanisms regulating carotenoid uptake or release from adipose tissue. This consideration provides further reason to consistently monitor habitual dietary L/Z intake and blood L/Z concentrations. These two measures are important as they may be used to provide insight into fluctuations in L/Z bioavailability, and influential factors such as diet and adiposity.

1.3.5.2 Lutein and zeaxanthin dietary intervention dosages

It remains unclear how different prescribed L/Z intervention dosages influences MPOD response. The aforementioned meta-analysis of RCTs by Ma et al.[33] reported that MPOD increased by 0.004 ODU for each additional 1 mg / day in L/Z/MZ supplementation in healthy individuals. [33] However, this dose dependent relationship was not observed in the six studies investigating different dietary dosages of L/Z in this review. [38-41, 43, 45] In the study by Kelly et al.[41], the control group was prescribed no change to diet, and four groups were prescribed a range of different L/Z dosages (0.26–1.61 mg/day L/Z) from egg. [41] Despite a range of dosages from a single food

source, no statistically significant within or between group differences were reported over the study duration. [41] Important to note is the difference in dosages between the dietary intervention trials and supplementation trials. In the meta-analysis of supplementation trials 15 of the 19 studies in healthy populations provided L/Z/MZ dosages above 10 mg per day. [33] These dosages are considerably higher than the doses provided by the dietary intervention studies included in this review (median dose was 0.98 mg/day, range 0.26–17.58 mg/day). Therefore, variation in habitual dietary L/Z intake is likely to exert a greater confounding influence on the effects observed after dietary modification providing lower additional doses of L/Z. Measurement habitual dietary intake must be considered in future investigations.

1.3.5.3 Dietary intervention food source

A statistically significant increase in MPOD from baseline was achieved after consumption of all of the intervention foods. However, only two prescribed interventions reported a significant MPOD response compared to the control group, and both were in populations with early AMD (50 years of age and above). The difference in MPOD between the intervention and control groups was 8.33% after 52 weeks with a small L/Z dose (1.59 mg/day) consumed with a fat source, [42] and 15.8% after 12 weeks with a much larger L/Z dose (17.58 mg/day) consumed without fat respectively. [44] It has been demonstrated that bioavailability is improved with co-consumption with fat. [57] These two studies in individuals with early AMD demonstrate an MPOD response achieved through prescription of L/Z containing foods with or without fat. Further studies demonstrating this relationship are needed in healthy individuals.

1.3.5.4 Dietary intervention duration

The time course of MPOD response with dietary intervention prescription remains unclear. An intervention duration of 12 weeks was the minimum length in which a statistically significant MPOD response was observed. The durations of studies that did not observe a statistically significant MPOD increase compared to baseline or to the control group were 12 weeks, [38, 41] eight weeks, [43] and five weeks. [40] The two studies in populations with AMD observed similar significant increases in MPOD compared to the control group over different intervention durations. In the study by Li et al.[44] the intervention group had a 16% greater increase over the 12 weeks compared to the control, whilst a 16% greater increase over 52 weeks compared to control was observed by Van Der Made et al.[42] MPOD was measured pre and post intervention in these two studies. With no interim measures it is not known when MPOD started to respond throughout the intervention.

The time course of MPOD response is also unknown in the studies in healthy populations in this review. Two studies that observed significant MPOD from baseline increases in the intervention group performed interim measures throughout the intervention. [37, 39]

In the first study with interim measures by Wenzel et al.[39], a significant increase from a baseline mean MPOD of 0.18 ODU was observed by week four for Group 1 (provided 0.28 mg L daily from egg), and was not significantly different at week eight or 12 compared to week four. Meanwhile, for Group 2 (provided 0.83 mg L/Z daily from egg) a significant increase from a baseline mean MPOD of 0.37 ODU was observed at week four and eight, with a further significant increase compared to week four and eight observed by week 12. [39] Group 1 and 2 were not compared, and baseline MPOD of the groups were significantly different. An increase in MPOD was observed in as little as four weeks, however further MPOD increase by 12 weeks was only observed with the higher L/Z dosage.

The second study with interim measures provided a dose of just 0.5 mg of L daily from avocado for 26 weeks. [37] In this study, a significant 23% increase from a baseline mean MPOD of 0.39 ODU was observed at 12 weeks, with no further change between 12 and 26 weeks. [37] No further increase in MPOD despite three more months of daily L intake may be due to what has been termed as 'MPOD saturation'. MPOD saturation is the suggestion that MPOD may be saturable, and that the threshold of saturation may be different between individuals. [58] This has been demonstrated in a cohort of 172 adults with AMD, mean age 70 ± 10 years, that were randomized to 3 groups. [58] Sixty subjects were supplemented daily for 12 months with 10 mg L and 1 mg Z, 66 subjects with 20 mg L and 2 mg Z, and 46 subjects with a placebo. Significant increase in mean MPOD compared to baseline and placebo was observed in both treatment groups by one month, and continued to increase until six months. Between six months and 12 months mean MPOD remained elevated but did not significantly increase compared to the 6-month measure. The absence of continued MPOD increase was suggested to be due to MPOD saturation. [58] Within the studies of this review, a significant MPOD response from baseline was been observed in as little as four weeks, and with a dietary intervention L/Z dosage less than that of the supplementation study [46]. Thus, the saturation theory may also have influenced the lack of MPOD response observed in four of the ten studies in this review. However, the potential influence of the saturation theory cannot be unpacked further as the studies in this review did not closely monitor habitual dietary L/Z intake. Measurement of habitual dietary L/Z intake is necessary to identify participants with regular consumption of L/Z rich foods that may influence MPOD saturability and the time course of MPOD.

1.3.6 Conclusion

No clear relationship between dietary L/Z interventions and MPOD response could be determined in this review. Appraisal of the studies identified that factors limiting the determination of any relationship include the lack of quantitative monitoring of habitual dietary L/Z intake over the study duration, and heterogeneity in study design. Heterogeneity in study design included variety of food source, L/Z dosages administered, intervention duration, participant characteristics, and inclusion of a control group. Future studies investigating MPOD response to dietary L/Z interventions should consider the use of a validated dietary intake tool designed to quantitatively measure dietary L/Z intake over the study duration.

1.3.7 Summary

The narrative review found no interpretable relationship between dietary L/Z interventions and changes to MPOD. Relative to research investigating the impact of supplemental L/Z and increases to MPOD, the number of studies investigating dietary L/Z interventions are limited and heterogeneous. [33] A more recent systematic review and meta-analysis reviewing the impact of L/Z intake from supplement or dietary intake on MPOD supports the findings of this narrative review. It was reported that no significant change was observed in MPOD when pooled studies investigated L/Z dosages less than 5mg/day. [59] The three studies pooled for this finding were three included in the Chapter 1 narrative review. The identified gap in understanding of the relationship between dietary L/Z interventions and MPOD change demonstrated the need for further exploratory studies addressing this relationship. In relation to the research framework applied in this thesis the review outcomes also indicated that criterion 6, clinical trials for dose-response efficacy, is not currently met when L/Z is provided through dietary intake. The lack of quantitative monitoring of habitual dietary intake was identified as a major factor limiting the determination of any dose-repose relationship in the narrative review. A quantitative value for habitual L/Z dietary intake was rarely and inconsistently reported across most of the studies. For those studies that did attempt to quantitively monitor habitual dietary L/Z intake, tools were not specifically validated to do so. This lack of habitual dietary L/Z monitoring was identified as a limitation as without appropriate monitoring it is unclear to what extent habitual dietary L/Z intake is influencing reported study MPOD outcomes. Thus, the key gap to be addressed in this thesis identified from this literature review was the need for development of quantitative dietary intake tool specifically designed to monitored habitual dietary L/Z intake. This new tool can then be applied to explore the relationship more effectively between dietary L/Z intake and MPOD.

1.3.8 Literature update since review publication

A literature search was conducted to update the narrative literature review on the 3rd August 2023. Using the search method described earlier (section 1.3.3) four studies met the inclusion criteria (Table 1-2) [60-63] The sample sizes and participant characteristics were similar to that described in section 1.3.4. Three of the four studies used HFP [60, 61, 63], and one used fundus autofluorescence to measure MPOD [62]. The intervention foods provided between 0.185 mg/day and 28.95 mg/day of L/Z/MZ from goji berries, egg, or a mix of fruit and vegetables (fruit: avocado, kiwifruit, orange, vegetables: lamb's lettuce, green beans, pumpkin, sweet corn). Three of the four studies performed a measure of dietary L/Z intake using either 24-hour diet recalls or 7-day diet diaries. [61-63] A dose-response relationship was still not apparent in these four studies. Whilst three of the studies quantitatively captured small periods of dietary L/Z intake, none of the studies used a validated tool to capture longer-term or habitual dietary L/Z intake over the whole duration of the study. The gap identified in the from the narrative literature review remained.

634 Table 1-2 Study interventions and outcomes, literature update

Author (date) [study quality]	Study design	Participant characteristics	Inclusion criteria	Intervention (mg L/Z/MZ per food serve)	Mean MPOD		Blood L/Z/MZ response			Method of monitoring habitual dietary intake
					Baseline (ODU ± SD)	Study end (ODU ± SD)	% Change from baseline	L % change from baseline	Z % change from baseline	
Zhang et al. (2021) [60] [Ø]	RCT, 12 wk	n 96 (70% female), 22 – 72 years	High myopia	G1: 10g goji berries (1 mg L, 10 mg Z) G2: supplement (1 mg L) G3: 20 g goji berries (2 mg L, 20 mg Z) G4: supplement (2 mg L) G5: control (0 mg L or Z)	G1: 0.42 G2: 0.37 G3: 0.39 G4: 0.47 G5: 0.43	G1: 0.50 G2: 0.42 G3: 0.53 G4: 0.55 G5: 0.45	G1: 18%* G2: 13% G3: 37%* G4: 18%* G5: 6%	Not monitored	Not monitored	Not monitored

Li et al. (2021) [61] [+]	Prospective, parallel-arm, unmasked trial, 12 wk	n 27 (70% female), 45-65 years	Healthy	G1: 28g goji berries (28.8 mg Z, 0.15 mg L) G2: supplement (6 mg L, 4 mg Z)	G1: 0.67 ± 0.06 (at 0.25°E), 0.16 ± 0.02 (at 1.75°E) G2: 0.68 ± 0.06 (at 0.25°E), 0.16 ± 0.02 (at 1.75°E)	G1: 0.76 ± 0.06 (at 0.25°E), 0.21 ± 0.03 (at 1.75°E) G2: 0.74 ± 0.06 (at 0.25°E), 0.19 ± 0.03 (at 1.75°E)	G1: 13% (at 0.25°E)[b], 31% (at 1.75°E) [b] G2: 9% (at 0.25°E), 12% (at 1.75°E)	Not monitored	Not monitored	24-hour diet recall (ASA24®): one between day 0 and 45, one between day 45 and 90
Schnebelen-Berthier et al. (2021) [62] [+]	Monocentre, double-blind, randomized trial, 16wk	n 99, (49% female), 18-55 years	Healthy, non-smoking, BMI ≤30, <4 servings/wk of high carotenoid, phytosterol, omega 3 foods in last 3 months.	G1: 2 x standard egg/day (0.12 mg L, 0.065 mg Z, 37.6mg DHA) G2: 2 x enriched egg/day (0.96mg L, 0.1mg Z, 134.4 mg DHA)	G1: 0.55 (at 0.5°E), 0.46 (at 1°E), 0.26 (at 2°E), 0.11 (at 4°E) G2: 0.56 (at 0.5°E), 0.47 (at 1°E), 0.26 (at 2°E), 0.11 (at 4°E)	G1: 0.56 (at 0.5°E), 0.47 (at 1°E), 0.26 (at 2°E), 0.12 (at 4°E) G2: 0.57 (at 0.5°E), 0.48 (at 1°E), 0.27 (at 2°E), 0.12 (at 4°E)	G1: 2% (at 0.5°E), 2% (at 1°E)[b], 0% (at 2°E) [b], 9% (at 4°E) [b] G2: 2% (at 0.5°E), 2% (at 1°E)[b], 4% (at 2°E), 9% (at 4°E)	G1: 15% [b] G2: 121% [a, b]	G1: 29% [b] G2: 65%[a, b]	7-day diet diary wk before study and wk before end of study - paper. Participants asked not to change their dietary consumption (other than eggs) and remove all foods rich in lutein (cabbage, spinach, flaxseed etc.)

Olmedilla-Alonso et al. (2021) [63] [Ø]	Clinical trial, 4 wk	n 29 (21 women) mean age 55.6 +/- 4.9	BMI: 20-30. cholesterol 3.9 – 6.5 mmol/L	G1: 1.8 mg/day from fruit (avocado, kiwifruit, orange) and lamb's lettuce G2: 1.8 mg/day from vegetables (green beans, pumpkin, sweet corn) and lambs lettuce	G1: 0.31 ± 0.12 G2: 0.37 ± 0.12	G1: 0.28 ± 0.10 G2: 0.38 ± 0.14	G1: -10% G2: 3%	G1: 52% [b] G2: 23%	G1: 9% G2: -7%	3 x 24-hour diet recalls at baseline and conclusion of study. Recalls completed by interview over 7 days (one weekend day), one in person and two over telephone.

Study quality assessed by ANDQCC for primary research: (+) relevant and valid study, low risk of bias; (Ø), relevant study, moderate or unclear validity and risk of bias [36]. **[a]** significant difference between groups at baseline $p < 0.05$, **[b]** significant MPOD increase from baseline $p < 0.05$, **[c]** $p \leq 0.001$, ***** significant MPOD change versus control group $p < 0.05$. ^ Zhang et al. [60] G3 significantly higher than G4 $p = 0.040$, no difference between G1 and G2. G3 significantly higher than G1 $p = 0.011$. Abbreviations: AMD, age-related macular degeneration; BMI, body mass index; °E, degrees eccentricity from macular centre; G, group; L, lutein; MPOD, macular pigment optical density; n, number of participants; NR, not reported; ODU, optical density units; %, percentage; SD, standard deviation; wk, week; Z, zeaxanthin.

1.4 Thesis aims

The literature reviewed throughout Chapter 1 has identified strengths and gaps in the body of research surrounding the relationship(s) between L/Z and the macula. Returning this to the nine criteria (see Figure 1-3), the plausible biological rationale for L/Z's role in health presents a strong case. This case centres around not only their studied reduction in AMD risk and severity but also ocular function (e.g. contrast sensitivity). [64] In addition to ocular health and function, there continues to be emerging research regarding the role of L/Z in cognitive function, and risk of conditions such as Alzheimer's disease. [65] Regardless of how important the possible benefits of L/Z may be, scientifically supported amounts of dietary L/Z for the maintaining or improving concentrations of meaningful biological markers in the body are yet to be determined. MPOD is one such biological marker that is used as a proxy marker for risk of AMD, and has emerging potential as a marker of cognitive function. [66] An understanding of the amount of dietary L/Z required to maintain or improve MPOD would provide evidence in support of a recommendation for daily dietary L/Z intake.

The conclusions from the review of literature, however, indicated that the dietary intake required to maintain or improve MPOD was unclear. In relation to the nine criteria, this conclusion indicated that criterion 6, when observing dietary interventions and intake, is not met with the available research. The MPOD of participants did not consistently increase in a dose-response manner or at all when a dietary L/Z intervention was prescribed. [67] The dominant confounding factor was an inability to monitor habitual dietary L/Z intake validly and quantitatively. The inability to validly capture habitual dietary L/Z intake has implications for evidence cited in support of criterion 4, cohort studies. A contributing factor to the inability to capture habitual dietary L/Z intake also relates to criterion 3, a food database local to the population of interest with known amounts of a bioactive constituent.

Therefore, the primary research question of this thesis was:

How can habitual dietary L and Z intake be validly and quantitatively estimated to investigate links to ocular health?

The aims of this thesis were to:

1. Develop and validate a method for quantitatively capturing habitual dietary L/Z intake.
2. Develop and validate a method to investigate whether blue light exposure from usual electronic device use impacts macular L/Z concentrations.
3. Identify an appropriate method to analyse L/Z concentrations in local foods to increase data available in the Australian FCTs.

The aims of the thesis were addressed through the following objectives:

1. Development and validation of two dietary screeners designed to capture habitual dietary L/Z intake over one week and one month respectively in Australian and UK adults.
2. Development and validation of a questionnaire to capture usual ED use behaviours in Australian and UK adults.
3. To investigate the associations between ED use, dietary L/Z intake and MPOD in healthy Australian adults, using the newly developed tools.
4. Investigation of an appropriate extraction method for analysing food L and Z concentrations suitable for building local Australian FCT.

These four objectives were addressed with four original research projects and address the identified evidence gaps relating to the 9-criteria and thesis research question, see Figure 1-3.

An Australian and UK research population and setting were selected for investigation throughout thesis was completed as part of a dual institution study program between these countries.

To answer this research question and appropriately address the thesis aims and objectives further literature was reviewed throughout Chapters 2, 3, and 5. The literature includes available dietary methodologies, biomarkers of L/Z intake, L/Z food composition data, blue light in relation to the macula, and electronic device use (Table 1-3).

Table 1-3 Literature themes reviewed and location throughout thesis

Broad topic (Thesis section discussed)	Sub-topics	Relevance (related thesis aim)
Estimation of dietary L/Z intake (2.1)	• Possible methods • Existing research • Options for a new tool • Biomarkers of L/Z dietary intake	Explores literature relevant to the development and validation an appropriate tool to estimate dietary L/Z intake (aim 1).
Blue light and electronic device use in relation to macular L/Z concentrations and health (3.1)	• BL exposure (sources and methods for capture) and relevance to the macula • Methods to measure MPOD	Explores literature relevant to valid measurement of MPOD, and the implications of ED BL exposure on MPOD status (aim 2).
Food composition analysis and data (5.1)	• Pre- and post-harvest factors impacting L/Z concentrations. • Food L/Z sampling and analysis methods • Current status of US, UK and Australian food composition data	Explores literature relevant to analysing and interpreting food composition data (aim 3).

Abbreviations: L/Z, lutein and zeaxanthin; US, United States of America; UK, United Kingdome; MPOD, macular pigment optical density; BL, blue light; ED electronic device.

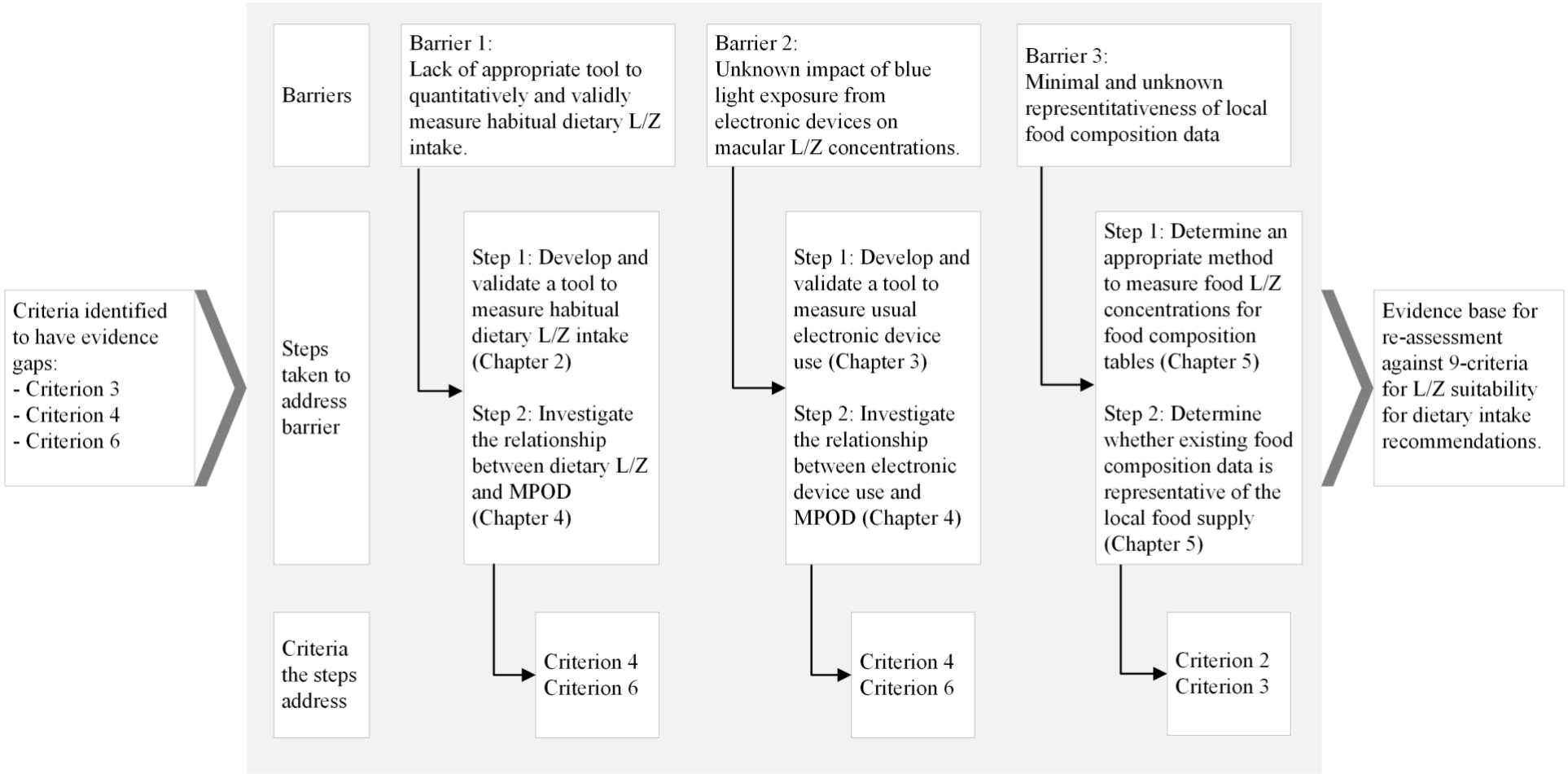

Figure 1-3 Barriers and steps to address lutein and zeaxanthin meeting the 9-criteria by Lupton et al. [2] to support a dietary intake recommendation

Chapter 2 Development and evaluation of the validity of a dietary lutein and zeaxanthin screener in Australian and United Kingdom adults

This chapter reviews literature relevant to the estimation dietary L/Z intake (section 2.1) and discusses the results of my original research study addressing thesis objective 1: the development and validation of two dietary screeners designed to capture habitual dietary L/Z intake over one week and one month respectively in Australian and UK adults (section 2.2 – 2.8). The literature explores factors that were considered in the development of the new dietary L/Z intake tool. These factors include types of dietary intake methods, methods utilised in existing research, options for a new tool, and relevant biomarkers of dietary L/Z intake (section 2.1).

2.1. Methodological review for estimation of dietary lutein and zeaxanthin intake

Investigating the relationship between the diet and a health condition is heavily reliant on appropriate assessment of the diet. Robust assessment of dietary intake can be difficult to perform due to the complexity of dietary intake behaviours. Dietary constituents and patterns of consumption are variable across days, population groups, and cultures. Assessment of the diet may involve broadly assessing whether a food is consumed or not. Alternatively, an assessment may be more specific with a focus on quantities of a particular group of foods, or nutrients. [68] Dietary assessment may be conducted through: individual or combined use of observation, a self-report tool, and biological markers. [69-71] Use of a biological marker, such as a serum concentration, is desirable due to its greater objectivity compared to observation or self-report. However, a biological marker cannot capture the actual foods consumed and is not always utilised due to practicality or feasibility issues such as cost. [68, 71] Thus, to date, many different methods to monitor dietary intake have been developed.

2.1.1 Methods to capture dietary intake in general

The overarching methods available to capture dietary intake, that is not specific to L/Z, are observation and self-report (Table 2-1). [69] Observational studies involve documentation of foods and drinks consumed via observer record and visual or weight assessment of plate waste across multiple eating occasions. Often deemed a more objective measure, this method may be influenced by observer error, or change in participant behaviour in response to observation. Observation is more likely to capture true intake compared with a self-report measure, therefore observation has commonly been used as the reference method to determine validity of other self-report methods. However, whilst capturing closer to true intake, observation may not capture usual intake due the observation environment or provision of foods not reflecting ‘natural’ settings for participants.

Additionally, observation is not often feasible due to issues such as lengthy observer training required, and the time and environment intensive nature of the method. [68, 72, 73]

The other overarching method to estimate dietary intake is known as self-report. Self-report dietary tools are a commonly used method and may be interviewer administered such as over the phone or in person, or self-administered such as by paper or electronic software. [71, 74]. Examples of self-report tools include a 24-hour diet recall, food frequency questionnaire (FFQ), and food records such as a 3-day or 7-day weighed food diary. [68, 71, 75] Each tool has its own strengths and limitations, but a similar limitation between all tools is the reliance on the user reporting their intake accurately. Accurate reporting can be difficult to achieve for numerous well investigated reasons such as: difficulty and inconvenience of estimating volumes or weights of a food, high inter-day variability in intake (also known as within-person random error), reactivity bias, and social desirability bias. [75-77] Reactivity bias refers to changes in dietary behaviours in response to using the tool, and can include social desirability bias. For example, when completing a weighed food record, consuming less food so that do not have to go through the 'effort' to weigh and record it. [77] Social desirability bias refers to participants tendency to overreport intake or volumes of foods perceived as 'good' and underreport foods perceived as 'bad'. What a participant perceives 'good' or 'bad' to be can be influenced by the context of the study, and participants have this tendency out of fear of judgment for their intake or desire to please investigators. [77, 78]

Each self-report tool has different recall timeframes and factors for consideration before use. Self-report tools that capture dietary intake over short timeframes are 24-hour diet recalls and food records. These tools aim to capture detailed information about the timing and quantity of all food and beverages consumed. The high detail of information is a benefit to these tools, and they can capture the complexity of dietary patterns. To compare between individuals or groups when using these tools, consideration of non-consecutive and repeat use is required. Non-consecutive and repeat use is needed to account for between day variation and day of the week effect. [68, 71, 75, 79] The day of the week effect refers to differences in dietary intake that may occur due to numerous social and cultural reasons, for example a work day versus weekend day. [79] A 24-hour recall is retrospective and aims to capture intake from the prior day (24-hours), for example midnight to midnight. Thus, when selecting a 24-hour recall the day of the week effect must be considered. A 24-hour recall relies on specific memory (rather than general). When they are pre-scheduled 24-hour recalls may incur reactivity bias, however less so when completed unannounced or randomly. The predominant type of error associated with 24-hour recalls is random error (versus systematic), and usually within-person random error. [80] A food record is prospective and usually

involves participants keeping detailed records of their intake through weighing or immediate record keeping for one, three or seven days. A food record is also associated with random error. However, without repeat, non-consecutive measures, a food record may also experience systematic error. While not reliant on memory, a food record is prone to reactivity bias and data quality can decline with increasing days of recording. [81]

A FFQ or screener are retrospective tools that capture usual intake over a defined period of time, for example one year. These two tools contain a finite list of foods and participants are to record how frequently the listed foods have been consumed over the specified timeframe such as one month, three months, six months, or 12 months. These tools may also contain a quantitative component for which participants record the serve size or number of serves (from a pre-determined serve size) they consumed. [75] A FFQ may be structured to capture total dietary intake, or just particular aspects of the diet. A screener is designed to capture particular aspects of the diet. These two tools are less impacted by reactivity bias, though they may still experience social desirability bias. These tools are useful for capturing foods that are consumed episodically and rely on general memory. The reliance on general memory can mean accurate tool completion is difficult for participants as general memory is cognitively complex and tasks such as mathematical averaging may be needed to report intake. [75, 82] These difficulties assist in explaining the systematic error associated these two tools. Systematic error is measurement results deviating from the truth consistently in a single direction. Systematic error can include both intake-related bias and person-specific bias. Intake-related bias is a function of true intake. High-intake of a food or foods that an individual consistently underreports is an example of intake-related bias. Person-specific bias relates to individual characteristics that will impact how intake is reported. A person-specific bias example is misreporting of intake related to social desirability or sociocultural norms. [83] These biases are important to consider when applying a FFQ or screener. FFQs have shown to underestimate true energy, protein, potassium and sodium intake, so cannot be relied on to produce absolute intakes of food and nutrients. However, they report good validity for ranking food and nutrient intakes within a population and can distinguish these intakes between subpopulations. [84-86] Each dietary intake tool is associated with particular biases, and thus selection of the tool most appropriate to the aims of the study is needed. To ensure tool appropriateness, continued assessment and development of tools that are specific to the dietary constituent and population of interest is needed.

Table 2-1 Comparison of available dietary intake methods

Method	Observation or self-report	Retrospective or prospective	Data capture timeframe	Types of error associated with method	Other considerations
Observer record	O	P	Short	Systematic error – environment not representative of 'real life'	Time-intensive, costly, highly detailed information captured
Plate waste	O	P	Short	Systematic error – environment not representative of 'real life'	Time-intensive, highly detailed information captured
24-hour diet recall	SR	R	Short	Within-person random error, reactivity bias, social desirability bias	Low cost, repeat measure often required, highly detailed information captured
3- or 7-day diet record	SR	P	Short	Within-person random error, reactivity bias, social desirability bias, systematic error ^	Low-moderate cost, highly detailed information captured
FFQ	SR	R	Long *	Systematic error, social desirability bias	Low cost, lower detailed information captured
Diet screener	SR	R	Long *	Systematic error, social desirability bias	Low cost, lower detailed information captured

*Timeframe of data capture is often long for example 12 months, but can be short. ^ Systematic error more associated with method when repeat measures. Abbreviations: FFQ, food frequency questionnaire; O, observation; SR, self-report; P, prospective; R, retrospective.

2.1.2 Existing tools used to estimate dietary lutein and zeaxanthin intake

Dietary L/Z intake has been investigated in many observational, epidemiological, and clinical trials. Despite these investigations, few dietary intake tools validated to monitor L/Z intake exist. Of the existing tools that have undergone validation testing, utilisation within in research studies, such as clinical trials, has been minimal. [67, 87-89] An appropriate tool is particularly important for clinical trials investigating impacts of L/Z dietary and supplementation interventions on outcomes such as MPOD and AMD progression. The tool is important as habitual dietary intake must be monitored to capture any potential influence on trial outcomes. [67]

Several different types of dietary measurement tools have been utilised to estimate dietary L/Z intake to date. Tools utilised include a short screener [90], short FFQ [91], 4-day weighed food diary [92], 7-day diet diaries, repeated 24-hour recall [93], dietary intake recall via interview [37,

94], and FFQs with a recall timeframe ranging from one to 12 months [95-97]. The outcomes of validation studies for a number of these tools are summarising in Table 2-2. Only the short screener and short FFQ were developed specifically to assess L/Z dietary intake. [90, 91] The screener asks participants to record per week frequency of intake of four foods; broccoli, eggs, corn and green leafy vegetables. [90] The responses are weighted by frequency of intake, bioavailability, and the food L/Z concentration to produce a score between 0 and 75. The only available data relating to screener validity was through a poster by Moran et al. [98] presented by the Macular Pigment Research Group. This poster data describes investigating the relationship between the screener and blood L/Z concentrations in 125 adults. The screener scores and blood L/Z concentrations were weakly linearly related with a correlation coefficient of 0.329, $r^2 = 0.109$, and $p < 0.001$. Although a significant correlation, it does not necessarily indicate that the screener is valid enough for the settings it is to be used in. The authors did not provide comment on whether this correlation indicated that the screener was valid.

The short FFQ validated in 87 females aged 20-25 years was deemed to be valid. [91] The FFQ had a recall timeframe of one month and included 10 fruits and 20 vegetables. Participants completed the FFQ via an interview process with a trained dietitian and photographic atlas assistance. A blood L/Z measure was taken on the day of FFQ completion. In the following days participants then completed a 7-day diet record using the same photographic atlas that was used to assist estimation of FFQ intake. Mean ± SD intake from the FFQ and 7-day record was 1,107 ± 113 μg/day and 1,083 ± 116 μg/day respectively. Intake between the two tools was significantly correlated ($r = 0.94$, $p < 0.0001$) and the Bland-Altman plot analysis indicated a mean difference (FFQ minus records) of -24.5 μg/day with 95% limits of agreement (LOA) from -50.6 μg/day to 99.6 μg/day. Dietary L/Z intake from the FFQ was also significantly correlated with plasma L/Z concentrations ($r = 0.76$, $p < 0.0001$). [91] It was noted by the authors that use of the same photographic atlas between the FFQ and diet records may have contributed to the close agreement. This study provides important insight into the valid and quantitative capture of L/Z intake. The FFQ only captured a subset of fruit and vegetables so is unlikely to be representative of habitual L/Z intake, and further detail on which of the 30 foods contributed to intake was not reported. The use of Bland-Altman plot analysis is a strength of this study and differed compared to many other questionnaires validation study. The Bland-Altman plot provided useful insight into the degree of agreement between the two dietary methods investigated. A limitation of this study is that it remains unclear whether the validity of the FFQ would remain high in males, or without a dietitian-assisted interview for completion. The requirement for the FFQ to be interview-administered would significantly decrease the cost-effectiveness and ease of use of a FFQ in both research and general populations. Another limitation of this study was the timing of the 7-day food record in the study

design. The 7-day record was completed in the 7 days after completion of the FFQ. The comparison of intake between the FFQ and record was therefore not comparing the same days of intake, and thus not reporting on whether participants recalled the same intake across the same period of time between two different methods. The close agreement between the two tools may therefore have resulted by chance, or may indicate FFQ validity and that dietary intake of these 30 foods in this population fluctuated minimally over the five observed weeks (4 weeks of FFQ, 1 week of 7-day food record). Study design could be improved by the 7-day food record occurring in one of the four weeks preceding the FFQ. Despite these limitations, this study suggests that with a questionnaire that captures a broader array of foods, it could be possible to capture habitual L/Z intake validly and quantitatively in adults.

Table 2-2 Dietary intake tool validation study comparison

Study	Population	Tool Comparison	Correlation Coefficient	Deattenuated correlation coefficient	p value
Moran et al. (2014) [98]	Adults, nationality unclear (n 125)	4-item screener recalling weekly intake and blood L/Z	0.33	0.11	<0.001
Cena, Roggi, & Turconi (2008) [91]	Italian adults (n 87)	30-item FFQ with 1-month recall timeframe, and 7-day diet record, and blood L/Z	0.94 [a] 0.76 [b]	-	<0.001 <0.001
Cho et al. (2008) [99]	American women (n 162) American men (n 110)	130-item FFQ with recall timeframe of 12-months and blood L/Z	0.23 [c] 0.38 [c]	- -	<0.05 <0.05

[a] Correlation coefficient between FFQ and food record. [b] Correlation coefficient between FFQ and blood L/Z. [c] Adjusted for age, body mass index, plasma cholesterol, and plasma triglycerides. Abbreviations: L/Z, lutein and zeaxanthin; FFQ, food frequency questionnaire; n, number of participants

Dietary L/Z intake has most commonly been monitored in observational and cohort studies. [13, 97, 99-102] A FFQ has been most commonly selected to monitor dietary L/Z intake, however most FFQs were not validated for quantification of L/Z. [100-102] Mixed outcomes were observed in the small number of studies that have considered validity of their selected tool to monitor L/Z (Table 2-2). In a cohort study by Cho et al. [99] in 2008, testing of FFQ validity to estimate L/Z Intake was performed over the duration of the 16-year study. The FFQ utilised was 130-items with a 12-month recall timeframe. Reported L/Z intake from a FFQ was correlated with blood concentrations collected before completing the FFQ. The observed correlation was 0.23 in 162 women and 0.38 in

110 men between energy adjusted L/Z intake from the FFQ and plasma L/Z adjusted for age, BMI, plasma cholesterol and plasma triglycerides. [99, 103] As will be discussed later in the thesis in more depth, a 12-month recall timeframe is unlikely to reflect a single time point of blood L/Z concentrations. The low correlations observed may have been better explored with use of multiple blood collections over the 12 months and an additional dietary intake method that captured L/Z to better understand this FFQs capacity to monitor and rank participants by dietary L/Z intake effectively. O'Neill et al. (2001), developed a 107-item FFQ with a recall timeframe of 3 months that included foods aimed at capturing intake of six carotenoids. These six carotenoids were α-carotene, β-carotene, β-cryptoxanthin, lycopene, L and Z. Concurrent validity through photographic atlas assisted 7-day diet records indicated that in 118 Irish adults the FFQ reported mean dietary L/Z intake more than 50% greater than records. [97, 104] Tan et al. [13] in 2008 used a 145-item FFQ modified from Willett et al. [95] with a recall timeframe of 12 months. Concurrent validity was tested via 4-day weighed food records on three occasions. Both short- and long-term reliability was tested with a follow up FFQ at 4-6 weeks and 12 months. The FFQ was reported to overestimate total energy intake and all nutrient intakes by 10-20% compared with weighed food records. [13, 92]. These validation and reliability data outcomes were not reported with consideration for L/Z. [13, 92]

Nineteen L, Z and MZ supplementation trials were systematically reviewed by Ma et al.[33] in 2016, and only seven attempted to estimate dietary L/Z intake at all throughout the study. Of these seven, three provided no information regarding the specifics of the dietary intake tool such as number of items or validation history. [15, 58, 105] One of the remaining four studies utilised the aforementioned L/Z screener developed by Moran et al.[98]. [90] One utilised a 100-item FFQ that did not consider L/Z in its validation. [49, 106] The remaining two studies utilised the same 150-item FFQ with a 2-3 month recall timeframe. [107, 108] This tool was not validated with consideration for L/Z. [109, 110] Although original validation did not include L/Z, the questionnaire had previously been reported as valid due to dietary intakes from the FFQ being found to be significantly correlated with serum L ($r = 0.28$) and serum Z ($r = 0.24$) in a study not designed to validate the FFQ. [111, 112]

Several studies have utilised dietary intake methods other than a FFQ or screener such as 24-hour diet recalls and diet diaries with a duration of recording ranging from 3 to 7 days. [37, 92-94] Due to the small number of days captured by these methods, repeat use is needed to make effective comparisons between individuals or groups. In the context of capturing L/Z intake, repeat use becomes even more important due to non-ubiquitous presence of L/Z across foods. An

understanding of day-to-day variability in L/Z intake in the population of interest is needed to make an informed decision about the minimum number of days that is likely to capture usual intake. Without this understanding of the tool and population, interpretation of observational or intervention study results is more difficult. The potential limitations of using these short-term dietary intake methods without prior validation in the population of interest is evident in prior research. Olmedilla-Alonso et al. [93] investigated L/Z and anthocyanin supplementation effects on MPOD over 8 months in post-menopausal women. The 72 women were split into three groups, one receiving a L/Z supplement (6 mg L, 2 mg Z), one receiving an anthocyanin supplement, and one receiving both the L/Z and anthocyanin supplement. Dietary L/Z intake measured by a 3-day food record was completed at baseline, 4 months and 8 months. A significant increase in serum L/Z concentrations was found in the two L/Z supplementation groups and a significant dietary intake increase from baseline in all three groups. Despite both L/Z supplementation and increased dietary L/Z intake, no significant increases in MPOD were observed. Additionally, no significant correlations between dietary intake and MPOD were found. No prior validity testing of whether a 3-day food record is representative of dietary L/Z intake was reported. Additionally, the potential impact of dietary L/Z intake in this study is unable to be determined without a control group to compare against. [93]

Many different tools have been utilised to attempt to estimate dietary L/Z intake. Few tools have been tested for their validity to estimate intake of L/Z, and those tools that have been tested for validity either return poor validity or are not representative of habitual L/Z intake.

2.1.4 Relevant biomarkers of dietary lutein and zeaxanthin intake

A method available to investigate the validity of a dietary intake tool is comparison to a relevant biomarker. Biomarkers relevant to dietary L/Z intake include blood L/Z, adipose tissue and MPOD. Methods to measure MPOD are discussed in greater detail later (section 3.1.2.5). This section will review literature relevant to blood, brain, and adipose tissue L/Z concentrations. In addition to having potential utility in the validation process of a dietary L/Z intake tool, the relationship between dietary L/Z intake and relevant biomarkers are also important to understand due to the influence these markers may have on reported relationships between dietary L/Z intake and MPOD. For example, adipose tissue may be a confounding factor in the relationship between dietary L/Z intake and MPOD. Therefore, to address criteria, such as criterion 6, a new tool to capture dietary L/Z intake needs to be robust enough to effectively investigate relationships between intake with markers such as MPOD, independent of other human physiological factors that may confound the

relationship. [2] Alternatively, the limitations of a dietary intake tool must be clearly understood so it is used in research settings in which it is appropriate and valid to do so.

2.1.4.1 Blood lutein and zeaxanthin

2.1.4.1.1 Bioavailability

The bioavailability of L/Z relates to criterion five of the research framework described in this thesis (Figure 1-1, page 31) and this criterion is clinical trials on metabolic processes. Metabolic processes may include digestion, absorption, activation, transport and excretion. Bioavailability of L/Z is an important factor as it will impact the outcomes of a dietary intervention exploring research criterion 6, clinical trials for dose-response and efficacy. Additionally, it helps inform how varying amounts of dietary L/Z intake consumed from different foods captured by a new tool may be expected to relate to biological markers such as blood L/Z and MPOD. Lutein and Z are fat soluble carotenoids. Their bioavailability is influenced by food processing and other food constituents consumed with them such as dietary fat, fibre and other carotenoids [56]. Upon consumption, mastication followed by swallowing and release of digestive enzymes allow accessibility for absorption. In the small intestine L/Z are emulsified with fat and incorporated into lipid micelles. These micelles are absorbed into intestinal enterocytes through both passive and facilitated diffusion. Apical membrane proteins shown to facilitate L uptake include SR-B1, and NPC1L1. [56, 113, 114] Once absorbed, L/Z are incorporated into chylomicrons within the enterocyte and transported to the liver via the lymphatic system. Xanthophylls have also shown to be associated with apolipoprotein A-1 on the basolateral membrane of enterocytes. [56] From the liver L/Z are packed into lipoproteins to be transported to other tissues in the body, such as the retina. Both L and Z have been shown to predominantly associated with high density lipoproteins (~50%), followed by low density lipoproteins (~35-40%) and very low-density lipoproteins (~8-10%). In vitro analysis using adult retinal pigment epithelial cell line 19 (ARPE-19) cells has shown Z uptake was most efficient from high density lipoproteins, while L was more efficiently delivered from low density lipoproteins compared to high density lipoproteins. Additionally, in the presence of increased β-carotene serum concentrations, L uptake into ARPE-19 cells was decreased while Z uptake remained unchanged. [115] Proteins proposed to play a role in L/Z uptake into the RPE are glutathione S-transferase P1 (GSTP1) for Z and Steroidogenic acute regulatory domain 3 (StARD3) for L. At all stages of digestion, absorption, transport, and storage, there is the emerging potential variability in efficiency related to genetic variability. [116-119] This growing research area is not the focus of this thesis but is another factor that will contribute to inter-individual differences in how participant reported dietary L/Z intake relates to biological markers of Intake such as blood levels and MPOD.

The degree of variability possible with L/Z bioavailability from food suggest that two individuals accurately reporting a daily L/Z intake of 4 mg/day may result if different circulating blood L/Z concentrations. Therefore, in the context of attempting to validate a new dietary L/Z intake tool, if blood L/Z was used as the comparative method to estimate tool validity, the tool could appear to over- or underestimate dietary intake despite both individuals having consumed the same foods. Therefore, with the understanding that the bioavailability of L/Z from foods can be highly variable between foods and individuals, relying solely on a biomarker such as blood L/Z to validate a new dietary L/Z tool may incorrectly over- or underestimate tool validity. A new dietary intake tool should look to be validated against both a relevant biomarker and an existing dietary intake method.

2.1.4.1.2 Plasma half-life of lutein and zeaxanthin

The plasma half-life of L/Z is another factor that may inform an appropriate process to validate a new dietary L/Z tool, and how this tool may relate to other biomarkers of interest such as MPOD. In particular, understanding the plasma half-life of L/Z provides insight into an appropriate length of recall timeframe for a dietary intake tool, especially if the aim is to relate the dietary intake to plasma levels. Additionally, it provides insight into how plasma L/Z may be expected to relate to dietary L/Z intake or MPOD.

Unfortunately, the L and Z plasma half-lives remain unclear and have been reported to be between 5 and 76 days. [120-122] In 10 healthy women, 23–43 years old, mean ± SD body fat of 33.7 ± 8.2 g/100 g, following a low carotenoid diet for approximately 80 days, the reported mean plasma half-life of L and Z was 76 (standard error of mean, ± 17) and 38 (standard error of mean, ± 7 days respectively. [120] The standard error of the mean (SEM) indicates that between-person variability in when the half-life for L and Z occurs is present. This study was rigorously performed with participants living at a metabolic research unit over the duration of the study, and low carotenoid dietary intake was provided. A limitation of this study is that a β–carotene supplement was administered throughout the study and thus it is unknown whether this may have impacted the half-lives reported of L/Z. Another limitation of this study is that habitual dietary L/Z intake prior to the commencement of the study was not captured. At baseline, the inter-person variability in blood concentrations of Z was low but high for L. With a reported mean half-life of L being as large as 76 days and no estimation of dietary L intake prior to study commencement, it is unclear how habitual L intake may have influenced these study outcomes.

In another study, 10 subjects with Type 1 Diabetes Mellitus with a mean ± SD age of 24 ± 6 years, and 8 healthy controls of 27 ± 3 years followed a low carotenoid diet for 21 days (<0.3 mg/person/day). No differences in estimated carotenoid half-life were reported between groups, thus for all subjects the mean estimated carotenoid half-life of L and Z was 20 (95% CI, 15-25) and

25 (95% CI, 18-32) days respectively. [121] A low carotenoid diet was maintained in this group through provision of a list of foods for participants to avoid, and recording of dietary intake daily to check compliance. Once again, L/Z intake prior to baseline was not captured and therefore the influence of diet on baseline blood L/Z cannot be interpreted.

Lastly, 8 adults, mean age 28.6 ± 7.9 years, were supplemented with 4.1 mg /day L and 8 adults, mean age 28.6 ± 4.8 years, were supplemented with 20.5 mg/day L for 42 days. By day 18 of supplementation plasma L concentration was at a >90% fraction of the steady state concentration for both groups. Subjects were followed for a further 25 days post supplementation cessation, L half-life was not significantly different between groups and ranged between 5 and 7 days. [123] Throughout the study subjects were given a list of L/Z rich foods to avoid and a 1-day diet record was completed three times per week to monitor intake. Once again, habitual L/Z Intake prior to the commencement of the study was not captured.

The half-life of L and Z after cessation of a supplement or following low carotenoid diet was not consistent between the aforementioned studies. The variability in L/Z half-life has been proposed to be related to between-study methodological differences and influences of physiological characteristics such as body composition, age, and blood cholesterol profiles. [120, 124] However, in the study by Burri et al.[120], body weight, body fat percentage, lean mass, and blood cholesterol and triglyceride concentrations did not significantly influence carotenoid half-life. The predictability of L/Z half-life remains unclear. However, from the studies observed it appears that after a change to dietary (rather than supplemental) L/Z intake occurred, plasma changes may observable between 3 weeks and 3 months. This broad timeframe supports the selection of a tool with a longer recall timeframe such as a screener, compared to shorter timeframe methods such as a 24-hour diet recall, as intake may be more likely to reflect blood concentrations. [75] Additionally, the broad timeframe suggests the use of blood L/Z alone as the reference method to determine validity of a new dietary L/Z intake tool may results in over- or underestimation of tool validity. Therefore, a new dietary intake tool should look to be validated against both and existing dietary intake method and blood L/Z.

2.1.4.2 Lutein and zeaxanthin in human tissues other than the macula

Other tissues that L/Z are deposited in are adipose and brain tissue [56]. Understanding other human tissues that may accumulate L/Z is important as it may influence the strength of any relationship attempting to be investigated between dietary L/Z intake and MPOD status. The brain is an identified tissue concentrated with L/Z that may support cognitive function. [66] Although concentrations are comparatively higher in the macular, L has been reported to be the most concentrated carotenoid in the brain and is positively associated with cognitive function and

performance. [125] A L specific binding protein, StARD3 has been identified to facilitate brain L uptake. [126] The mechanism of action for L/Z in the brain remains unknown, however one hypothesis is that it is similar to that reported for the macula, antioxidant and anti-inflammatory. [66] L may be well positioned perform the role of oxidation prevention of important brain polyunsaturated fats, such as docosahexaenoic acid (DHA). L may be able to perform this role due to localisation in membranes rich with polyunsaturated fats and polar end groups that may allow for orientation that is perpendicular or semi-perpendicular to a membrane surface. [127] Interestingly, MPOD has been identified as a surrogate marker of brain L concentrations and related to cognitive performance. [128, 129] Methods to measure MPOD are discussed in greater detail later (section 3.1.2.5). Brain tissue samples are highly inaccessible and therefore not a feasible biomarker to regularly compare to dietary L/Z intake. However, understanding that L/Z is present in brain tissue positions brain L/Z concentrations as a confounding variable to be aware of when attempting to investigate associations between dietary L/Z intake and other biomarkers such as blood L/Z or MPOD.

Adipose tissue is another tissue that has been identified to contain L/Z. Adipose tissue has potential to be utilised as a marker of dietary L/Z intake. Additionally, adiposity may be confounding factor when attempting to determine the relationship between dietary L/Z intake and other biological markers such as blood L/Z or MPOD. In a study of 12 women and 13 men, L/Z were significantly more concentrated in the abdomen (456.3 pmol / mg) than the buttocks or thighs (227 pmol / mg and 268.5 pmol / mg respectively). [130] In this study L/Z dietary intake measured by the 100-item Healthy Habits and History FFQ was not significantly correlated with individual and combined abdominal, buttock, thigh or serum L/Z concentrations. Individual and combined serum L/Z was significantly correlated with abdominal L/Z (combined L/Z $r = 0.535$) but not buttock or thigh. The lack of relationship between dietary intake and adipose L/Z concentrations suggest that adipose tissue is not currently a viable biological sample to gauge habitual dietary intake. However, it indicates that adiposity may be a confounding factor when attempting to relate dietary intake with other biological markers such as plasma L/Z or MPOD.

Adiposity as a confounding factor is corroborated with the mixed outcomes to date of research investigating associations between adiposity, blood L/Z or MPOD in healthy adults across a range of ages, BMI, and body fat levels. [55, 131-136] In studies where men and women have been combined for analysis, body fat percentage has been reported to be uncorrelated with MPOD [134], or significantly negatively correlated with MPOD. [131, 133] At times, body fat percentage has been reported to be uncorrelated with plasma L/Z or dietary L/Z intake [131, 134], or significantly negatively correlated. [133] Many studies have analysed men and women separately and found

conflicting results. In men, body fat percentage has been reported to be significantly negatively correlated with MPOD, but uncorrelated with plasma L/Z. In women, body fat percentage has been reported to be uncorrelated with MPOD and significantly negatively correlated with plasma L/Z. [55] Biopsy of adipose L or Z concentrations have shown positive correlations with plasma L/Z in combined cohorts of men and women. [130, 132]. For MPOD, positive correlations with adipose L concentrations have also been identified in men but not women. [132] The use of BMI is common due to ease of measurement. In combined sex cohorts, BMI has been reported to be both uncorrelated or significantly negatively correlated with plasma L/Z and MPOD. [133, 134] When separated by sex, BMI has been reported to be negatively correlated with plasma L/Z for women but not men, but BMI negatively correlated with MPOD in men and not women. [55, 132] The differences in outcomes for whether BMI is correlated with MPOD, dietary L/Z intake or blood L/Z may be related to BMI not being an accurate reflection of adiposity. A reason these studies present inconsistent results may be that none of the studies captured weight history of their participants or determined if participants were in energy balance. Changes to weight or adiposity status, or being in a state of energy restriction may have influenced study outcomes. [137]

The importance of capturing weight 74istoryy and energy intake is supported by the study from Kirby et al. [136]. This group conducted a 12-month weight-loss RCT and investigated the interactions between adiposity, MPOD, and plasma L/Z. [136] In this study 104 adults with a BMI ≥ 28 kg / m^2 were randomised to a control group or weight-loss intervention that involved eating to a low-fat low-energy meal plan (dietitian prescribed), one hour of exercise per day, motivational lectures, and a weekly weigh in. Body fat percentage was measured by DEXA, MPOD by HFP and dietary L/Z intake with the Scottish Collaborative Group semi-quantitative FFQ with 12-month recall timeframe. There were no significant changes within or between groups for body fat percentage, MPOD, dietary L/Z intake, or serum L/Z. In a subgroup of participants that did lose weight a significant positive correlation between serum L (but not Z) and changes in BMI or body fat (kilograms and percentage) were found, correlation coefficients ranged from 0.51 to 0.73. [136] This subgroup analysis indicates that energy restriction and loss of adipose tissue can result in increases in serum L. It remains unknown how increases in adipose tissue may influence blood L/Z. However, the outcomes of this study highlight the importance of capturing weight and dieting history of participants when attempting to investigate the relationship between dietary L/Z intake and plasma L/Z or MPOD.

The inconsistent outcomes of the research to date indicate the adipose tissue is not presently a viable indication of dietary L/Z intake. Therefore, it cannot be used in determining the validity of a

new dietary L/Z intake tool. However, it appears that there is a relationship between adiposity and other markers that may reflect dietary L/Z intake such as blood L/Z and MPOD. Therefore, when attempting to relate dietary intake to blood L/Z or MPOD, adiposity is a variable that should be measured. Additionally, the measure of adiposity should be from methods more specific than BMI, such as dual-energy x-ray absorptiometry (DEXA) or bioelectrical impedance (BIA).

2.1.3 Background for development and validation of a new L/Z dietary intake tool

Selection and validation of an appropriate tool when attempting to capture L/Z dietary intake is complicated by their non-ubiquitous presence across all foods. The foods L/Z are concentrated in are green leafy vegetables, corn, eggs, cruciferous vegetables and select nuts, seeds, and fruits. [138] The variable concentration of L/Z across foods means day-to-day intake has potential to be highly variable. Attempts to validate tools with longer recall timeframes (e.g. 3-12 months) against a once-off measure of blood L/Z concentrations may be difficult due to this non-ubiquitous distribution of L/Z intake across foods. For example, low correlation between FFQ and blood could occur with a high average daily L/Z intake calculated from a 12-month FFQ in which high consumption of L/Z occurred in the first 3 months of the year, but the low blood measure was taken at the 12-month mark. Tools with shorter recall timeframes also have potential to correlate poorly with blood L/Z concentrations. For example, a high blood L/Z concentration being compared against a 7-day diet diary in which L/Z consumption was recorded as low, but intake was high the week prior.

2.1.3.1 Characteristics of a purposely designed lutein and zeaxanthin screener

To address the limitations of prior studies investigating the validity of dietary L/Z measurement a new tool is needed. A screener looking specifically at L/Z foods would be a viable a tool that could address the non-ubiquitous spread of L/Z across foods. A screener with a recall timeframe of one month has shown potential in the study by Cena, Roggi, & Turconi [91], and is likely to reduce memory recall bias associated with retrospective methods such as a screener. A short recall timeframe limits the potential ability to capture usual intake over long timeframes such as a year. Thus, similar to tools such as 24-hour recalls and diet diaries, repeat use of the screener over longer timeframes could be implemented to meet needs for long timeframe capture.

To quantitatively capture intake with a new screener, reporting of intake could utilise frequency of standardised portion sizes, or request self-report of usual portions consumed. Specific to dietary L/Z intake, it is unclear which of standardising portions sizes or requesting self-reported portions will best support accurate reporting. Some research suggests providing standardised portion sizes in a

screener may reduce reporting error comparative to asking questions about portion sizes. [139] Therefore, use of standardised portion sizes is a logical starting point that can be reassessed as needed.

A method to test the validity of a new screener would need to be carefully considered to avoid over or underinflation of the screener's utility. Ideally, an objective measure, such as blood L/Z, and a reference dietary intake method would be used to compare against reported L/Z intakes of a new tool. The reference dietary intake method should be selected to have different types of measurement error compared to a screener in order to not overinflate validity. [140] Therefore, when developing a screener, methods subject to different error, namely random and reactivity error, would be appropriate. Methods with such error include 24-hour diet recalls and food records. Correlation coefficients between two dietary intake methods may be higher when a reference method is used for 8–14 days in comparison to 1–7 days. [139] A 24-hour recall or food record requires repeat measurement to ensure an adequate number of days are recorded. An additional consideration is that continuous data collection has been reported to decrease correlation coefficients when it is more than five days. [139] Food records ask participants to record intake continuously and thus may be subject to this reduced data quality of continuous recording. [81] A 24-hour diet recall could be an appropriate reference as data collection can be repeated on non-consecutive days, and spread randomly over a new screener recall timeframe of interest. As discussed later (section 2.6) a blood L/Z was not able to utilised in the evaluation of the new L/Z dietary screener at the time of this thesis due to COVID-19 pandemic research restrictions.

2.1.3.2 24-hour diet recalls as a reference method

A 24-hour diet recall may be completed via telephone interview, in-person interview, or more recently through online platforms. [140, 141] An available and validated online format for completion of the 24-hour diet recalls is the 2016 Australian version of the online Automated Self-Administered 24-Hour Dietary Assessment Tool (ASA24®), developed by the National Cancer Institute, Bethseda, MD. [141] The ASA24® has demonstrated acceptable validity for reporting for energy intake in over 1000 adults (50–74 years), underreporting energy intake compared to recovery biomarkers by just 12–17%. [142] Specific to L/Z, outcomes of a validation study in over 600 women (45–80 years) indicated four ASA24® recalls completed over 15 months was poorly correlated with plasma L/Z (<0.45, exact correlation outcome not reported), and had low reproducibility between measures (adjusted rank class correlation 0.2). [143] This outcome suggests more than four repetitions of a 24-hour diet recall may be needed to capture intake appropriate for comparison against a new screener.

2.1.3.3 Ensuring accuracy of data collection with dietary intake methods

Underestimation of dietary intake when using self-report tools is an established concept. [140, 144] To identify unrealistic underestimation or overestimation of dietary intake methods such as the Goldberg cut-off can be used. [144, 145] The Goldberg cut-off represent the lower 95% confidence limit of the ratio of mean reported energy intake (rEI) and estimated basal metabolic rate (eBMR). The cut-off is the ratio at which it is statistically unlikely that the rEI is representative of habitual intake or a low intake obtained by chance. The cut-off value selected considers the number of participants observed and days of intake captured. A limitation of the Goldberg cut-off is the assumption that participants are sedentary, thus identifying underreporting in highly active individuals or overreporting is not possible without additional participant information. Additional information may include physical activity levels, weight change goals, and indication of whether the reported intake is 'normal' for the participant. Mean rEI for each individual participant requires use of a dietary intake measure from which energy intake can be calculated, for example a 24-hour diet recall. Participant eBMR requires collection and calculation of participant weight, height, and age with the Schofield estimation equations. [146] The ratio of mean rEI on eBMR can be compared to Goldberg cut offs to assess the accuracy of participant recall and identify over or underreporting. [144, 145] Participants with a ratio assessed as below the Goldberg cut off or grossly far above can then be cross-checked with their weight-related goals, physical activity, reasons provided for unusual intake days, and number of recalls below eBMR. In assessing the validity of a new dietary L/Z screener, utilisation of the Goldberg cut-off in conjunction with additional participant information would be appropriate and strengthen study outcomes.

2.2 Publication details

Sections 2.3 to 2.7 of Chapter 2 include the manuscript published in Nutrition Research (Journal Impact Factor: 4.5; Quartile 2). Numbering of tables, figures, and references are presented as part of the whole thesis and as such numbering is different to that of the submitted work. No other text in section 2.3 to 2.7 is different to the submitted manuscript.

N. K. Fitzpatrick, S. Capra, A. Shore, D. Briskey, S. Jackman, J. Bowtell, Chachay V. Newly developed dietary assessment tools for lutein and zeaxanthin are correlated with 24-hour diet recalls, but are not a valid measure of intake in Australian and United Kingdom adults. Nutrition Research. 2024;122:68-79. doi: 10.1016/j.nutres.2023.12.010

2.3 Introduction

The two carotenoids, lutein and zeaxanthin (L/Z), belong to a subgroup of non-vitamin A forming carotenoids known as xanthophylls. [25] Lutein and Z are not found ubiquitously across all foods. Foods rich in L/Z include leafy vegetables, broccoli, corn, eggs and goji berries [44, 138]. The ratio of L to Z is variable between foods. For example, green leafy vegetables may have 17 times more L than Z. [147] Comparatively, orange capsicums may be dominant in Z, with five times more Z than L. [148] In humans, L/Z have shown direct and indirect antioxidant functions, such as quenching singlet oxygen species and blue light absorption. [25] As such, dietary and supplemental intake of L/Z have been investigated for their role in ocular function, cognitive function, reducing risk of Alzheimer's disease, and reducing risk and severity of age-related macular degeneration. [10, 65, 66]

Populations in the highest percentile of dietary intake (upwards of 3 mg/day) or consuming a L/Z supplement (10 mg L/2 mg Z) were shown to have reduced risk or severity of AMD. [12-14] However, habitual dietary L/Z intake in recent observational, epidemiological, and clinical studies, was often not monitored or was captured with tools not specifically validated for L/Z. [13, 33, 67, 149, 150] Previous attempts to validate the measurement of dietary L/Z intake have been either unsuccessful or not specific to L/Z, for example, capturing total intake of many different carotenoids rather than L/Z exclusively. [143, 151-154] The current lack of specific and valid tools to quantitatively monitor habitual dietary L/Z intake is an identified barrier to advancing understanding of the diet-disease and dose-response relationships between L/Z and macular health. [67]

Methods to capture dietary intake most commonly rely on self-report and include tools such as the 24-hour diet recall (24DR), screeners, and food frequency questionnaires (FFQ). [75] These tools, although cost-effective and low burden for respondents, have well established validity and reliability limitations. [69, 70] One limitation is their reliance on accurate recall of intake by the respondent. Accurate reporting is limited by difficulties in estimating volumes or weights of food, high inter-day intake variability, and social desirability bias for certain foods. [75, 76] Developing new tools and improving existing ones is an active area of research to assist the understanding of diet-disease relationships, especially when the focus is on specific food constituents such as L/Z.

A screener is a type of diet assessment tool designed to capture a specific or small number of nutrients, and is thus appropriate for capturing episodically consumed dietary constituents. [75, 155] The non-ubiquitous presence and varied concentration of L/Z across foods increases the likelihood of episodic consumption. [138] This report describes the development and validation of a dietary screener designed to quantitatively capture habitual L/Z dietary intake for use in epidemiological and intervention studies. Two formats of an L/Z screener were developed; one with a recall

timeframe of a month (monthly screener - MS), and the other a week (weekly screener - WS). The aim of this study was to develop the L/Z screeners and investigate whether daily dietary L/Z intake measured by the screeners was valid with agreement within 0.25mg/day compared with intake measured from multiple 24DRs in adults residing in Australia and the United Kingdom (UK). Validity was tested by Bland-Altman plot analysis. [144, 145] These screeners are the first tools designed specifically for L/Z and address an identified gap of questionnaire tools needed to advance the understanding of the diet-disease relationship between L/Z and macular health. [67]

2.4 Methods and materials

Procedures for this study were in accordance with the Declaration of Helsinki and were approved by the University of Queensland Low and Negligible Risk ethics committee, and the Sport and Health Sciences ethics committee at the University of Exeter (#2020001764). All participants provided written informed consent.

2.4.1 Screener development

Two formats of a L/Z screener were developed with differing timeframes based on L/Z plasma half-lives, applicability to typical intervention trial lengths, and reduction of memory recall bias. [75, 120, 121, 141] Plasma half-life of L/Z has been reported to be between 5-76. [120-122] Therefore, recall timeframes of one and four weeks were considered to increase the potential that the screener would closely reflect circulating plasma L/Z levels. Five factors were considered when developing the screeners: timeframe of participant recall [75, 82, 120, 121], reference food composition tables (FCT) [138], foods to include, serve sizes [156], and frequency of intake. After initial development, an internal test of face validity was conducted with volunteers. [157]The MS and WS both contained 91 food items with defined serve sizes. Reference serve sizes were listed in both a volumetric and gram weight, for example ‘1 apple (165g)’. Participants could report frequency of food serves per week or per month for the MS, and solely per week for the WS. The FCT from the United States of America [138] and Australia [158] were used to identify foods rich in L/Z. Foods with more than 100 μg/100g of L/Z were prioritized for inclusion in addition to twenty foods with little or no L/Z. The inclusion of low L/Z foods aimed to reduce social desirability bias by increasing the range of foods reported. [77] The 91 food items were a mixture of cooked and raw foods, and included: 25 fruits, 39 vegetables, six grains, 12 meat and meat alternative foods (for example, nuts, seeds, and legumes), three dairy and alternative foods (for example, a calcium fortified soy beverage), and six discretionary foods (for example, chocolate). Discretionary foods were defined as per the Australian Guide to Healthy Eating. [156] One question asked participants to report the types of supplements being consumed in the last month (if any). The MS also included

a set of socio-demographic and anthropometric questions, and three questions about change in current dietary patterns compared to one, five, and 10 years ago. Lastly, the MS contained an open-ended question for respondents to note any other comments. The screeners were hosted on Checkbox Survey® for Australian participants and Qualtrics XM® for UK participants. See the supplementary materials.

2.4.2 Recruitment

A convenience sample of adults residing in Australia and the UK was recruited via electronic and paper advertisements between August 2020 and November 2021. Eligible participants were healthy adults, 18 years or over, able to complete online questionnaires. Exclusion criteria were no English language literacy, and visual, hearing, or physical impairment that prevented online questionnaire completion.

2.4.3 Data collection

Participants completed eight (two per week) 24DRs, four WSs, and two MSs over four weeks (Figure 2-1). The 24DRs were completed via the 2016 Australian version of the online Automated Self-Administered 24-Hour Dietary Assessment Tool. [141, 159, 160] The day for 24DR completion was randomly allocated by computer generated schedule at baseline within the constraints that two of the eight recalls were scheduled for weekend days, and the remainder for weekdays. The WS was completed at the end of each week. The MS was completed at baseline (MS1) and again at the end of week four (MS2). Participants were notified by email on the day a recall or screener was to be completed.

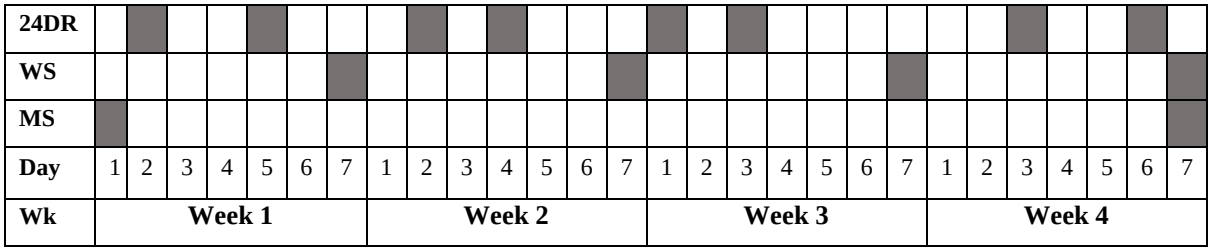

Figure 2-1 Dietary lutein and zeaxanthin screener validation study protocol in which (n = 103) healthy adults were asked to complete eight 24-hour diet recalls (2 per week on randomly assigned days, 2 of which included weekend days), 4 weekly screeners, and 2 monthly screeners over a 4-week period. 24DR, 24-hour diet recall; MS, monthly screener; WS weekly screener, MS monthly screener..

2.4.4 Lutein and zeaxanthin intake derived from the screeners

For each tool, total intake of L/Z from each individual food was calculated: (grams of food serve × number of serves reported) × (μg L/Z per gram of food) (1)

As Australia and the UK do not have comprehensive data for L/Z in their FCT, the µg L/Z per gram of food was obtained from the best matching food value listed in the United States Department of Agriculture (USDA) FCT [138]. Mean daily L/Z intake from foods in the MS1 and MS2 were calculated by dividing the sum L/Z from the month by 28. Mean daily L/Z intake from the combined weekly screeners (CWS) was calculated by dividing the total L/Z intake summed from all WSs combined, by the number of days captured from the CWSs. Supplemental L/Z intake was not incorporated as part of mean daily L/Z intake.

2.4.5 Lutein and zeaxanthin intake derived from the diet recalls

The Automated Self-Administered 24-Hour Dietary Assessment Tool output includes many parameters such as energy, macro- and micronutrients, but does not include L/Z. [141, 159, 160] Therefore, L/Z intake was calculated using a custom routine written in R (R Core Team, 2013). [161] The code utilizes word matching functions to link foods reported in the 24DR with the USDA FCT. Code outputs were screened for mismatches or missed foods and manually corrected. The total L/Z from all recalls was divided by the number of recalls completed to determine a mean daily L/Z intake.

2.4.6 Sample size

Dietary intake of L/Z using an L/Z specific FFQ or screener with a monthly or weekly recall timeframe has not been studied to date. Thus, a standard deviation of L/Z intake over this timeframe was unavailable for sample size calculation. As outlined in the documentation regarding the development of the Australian nutrient reference values, an intake coefficient of variation of 10% in the healthy population of interest is assumed. [162] The non-ubiquitous spread of L/Z in foods may indicate greater variability of intake. With more variable nutrients, a coefficient of variation of 15% is assumed. [162] Therefore, to capture the 15% coefficient of variation of dietary L/Z intake, a minimum of 30 participants was deemed required. Accounting for 20% participant attrition, a sample size of at least 36 participants per country (Australia and the UK) was determined.

2.4.7 Data management

The ratio of mean energy intake from the 24DRs to estimated basal metabolic rate were compared to the Goldberg cut offs to assess the accuracy of participant recall and identify over or underreporting as per methods described elsewhere. [144, 145] As shown in Figure 2-2, participant datasets were removed for identified over or underreporting using the Goldberg cut offs in combination with review of any participant reported reasons for unusual eating days and weight

related goals such as weight gain or loss. For the Australian and combined cohort Bland-Altman plot analysis, participants with fewer than eight 24DR or four CWSs were removed. For the combined cohort analysis of four CWSs and eight 24DR, the calculated intake difference between the tools was not normally distributed even after logarithmic base 10 transformation, except when an outlier participant reporting a difference between tools of 11.96 mg/day was removed. Results of the Bland-Altman plot analysis are presented with this outlier participant removed. For the UK cohort, participants were only removed if fewer than six 24DR or three CWSs were available. This increased the data available for analysis substantially as only eight participants completed all four WSs and eight 24DRs. The comparison between six 24DRs and three CWSs was deemed appropriate as the Australian cohort showed no significant difference in intake between six or eight 24DRs, and three or four CWSs.

2.4.8 Statistical analyses

Statistical analysis was conducted using SPSS (28.0). [163] Results are presented both combined and individually for the Australian and UK cohorts. Data normality was tested with the Shapiro-Wilk test. Differences between cohort participant characteristics and L/Z intake were tested with a Chi-square test, two-tailed independent samples t-test, or Mann-Whitney U-test. As intake of L/Z from each individual food was calculated, percentage contribution to total L/Z intake of each food group and individual food was calculated. An independent samples t-test for difference of means of the dietary L/Z intake reported between each of the tools was conducted. The outcomes were not significant; thus no assumptions were violated for a Bland-Altman plot analysis. To determine validity, a Bland-Altman plot analysis of the mean daily L/Z intake was performed to compare between the 24DR, MS2, and CWSs. [164, 165] The MS2 was used such that the timeframe in which L/Z intake was recalled was aligned with intake reported from the diaries. Pre-determined limits of agreement (LOA) did not exist on which to benchmark validity of the screeners. Informed by prior research, validity was therefore determined by whether the agreement with 24DR intakes was such that the screeners would have utility to detect changes in habitual intake at values that have been reported to impact macular L/Z concentrations in intervention studies. Dietary or supplemental intervention trials have reported providing as little as 0.5 mg/day L/Z, and observe change to macular concentrations. [33, 67] Therefore, the 95% LOA needed to be equal to or less than ±0.25 mg/day to adequately capture any impactful fluctuations in habitual dietary intake. Cronbach's alpha and two-way mixed effects model absolute intraclass correlation coefficient was performed for test-retest reliability between the MS1 and MS2. Normally distributed data is presented as mean ± standard deviation (SD) and non-normally distributed data as median and 25^{th} to 75^{th} percentile. Results were considered statistically significant at $p < 0.05$.

2.5 Results

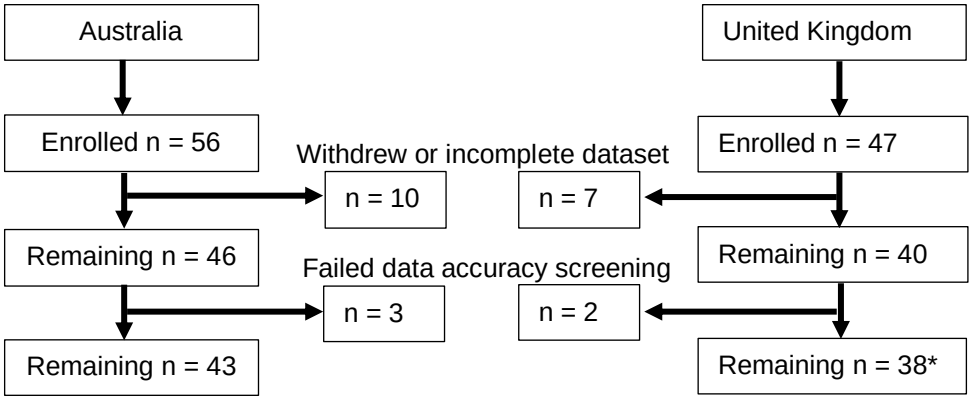

Figure 2-2 Participant flow chart of dietary intake study completion
In the Australian cohort 56 participants enrolled, 10 had incomplete data, and three failed data accuracy screening so 43 remained. In the United Kingdom cohort 47 participants enrolled, 7 had incomplete data, and two failed data accuracy screening so 38 remained. N = indicates the number of participants. *Indicates missing Monthly Screener 2 data for all United Kingdom participants

2.5.1 Participant characteristics

Fifty-six Australian and 47 UK adults enrolled in the study. Ten Australian participants and seven UK participants withdrew or failed to complete the required screeners and 24DRs (Figure 2-2). The median age of Australian participants was 25 (25 – 29) years, 73% were female and 64% had a tertiary education (Table 2-3). The median age of UK participants was 46 (40 – 50) years, 98% were female, 77% had a tertiary education. The age and tertiary education status of the UK participants was significantly higher than the Australian cohort, $p < 0.001$. The analysis of UK screeners and 24DRs was a female only cohort as the only male participant in the UK cohort did not meet the Goldberg cut offs and was removed.

Table 2-3 Participants characteristics of Australian and UK healthy adults.

	Australian (n = 56)	UK (n = 47)	Combined (n = 104)
Age, y	27 (25 – 29)	46 (40 – 50) *	33 (26 – 48)
Sex, female	73%	98%	85%
BMI, kg/m^2	24 (22.6 – 26.5)	24 (22.5 – 30.7)	24 (22 – 28)
Physical activity, hours/week	7 (4.9 – 9.0)	6 ± 3.9	7 (4 – 9)
Education, tertiary educated	65%	77%[a]	84%

Abbreviation: BMI, body mass index. Data are presented as median (25^{th} – 75^{th} percentile), mean ± standard deviation, or a percentage.. * Parameter significantly different between cohorts, $p < 0.001$.

The median daily L/Z intake reported from each of the tools ranged from 2.4 to 3.3 mg for the Australian cohort and 2.6 to 3.7 mg for the UK cohort (Table 2-4). Within a cohort, daily dietary L/Z intake captured by each tool was significantly correlated (Table 2-5). The strongest correlation

was in the Australian cohort between the MS2 and CWSs, R = 0.83, R^2 = 0.75 (p <0.001). There was also strong correlation between the Australian MS1 and MS2, R = 0.81, R^2 = 0.75 (p <0.001). The weakest correlation was between the CWSs and 24DRs in the UK cohort, R = 0.62, R^2 = 0.11 (p = 0.002).

Table 2-4 Daily lutein and zeaxanthin intake from the monthly diet screener, 4 combined weekly diet screeners and 8 combined 24-hour diet recalls in Australian and UK healthy adult cohorts individually and combined.

Tool	Australia		UK		Combined	
	n	Intake	n	Intake	n	Intake
MS1	49	3.2 (2.2 – 5.3)	38	3.7 (2.1 – 5.4)	87	3.4 (2.1 – 5.3)
MS2	42	2.7 (1.7 – 3.5)	-	-	-	-
4 CWS	35	2.8 (2.1 – 4.3)	15	2.8 (1.6 – 3.9)	50	2.8 (1.9 – 4.3)
8 combined 24DR	32	2.4 (1.6 – 3.1)	9	2.6 ± 0.76	41	2.4 (1.6 – 3.1)

Abbreviations: MS1 monthly screener 1, MS2 monthly screener 2, CWS combined weekly screeners, 24DR 24-hour diet recalls, Intake data presented as median (25^{th} – 75^{th} percentile) or mean ± standard deviation mg / day of lutein and zeaxanthin.

2.5.2 Comparison of screeners with 24-hour diet recalls

The Bland-Altman plot analyses indicated poor agreement of daily L/Z intake between the screeners and 24DRs, with modest mean differences but large 95% LOA (Table 2-5). In the Australian cohort, between the CWSs and MS, the CWSs had better agreement with the 24DRs. Participants were more likely to report higher L/Z intake with the CWSs compared to the 24DRs, with a mean difference of 0.51 mg/day and 95% LOA of -1.46 to 2.49 mg/day. The Bland-Altman plot analysis between the MS2 and eight combined 24DRs indicated a mean difference in daily L/Z intake of 0.33 mg/day and 95% LOA of -2.91 to 3.58 mg/day (Table **3**). Seven participants reported a mean L/Z intake above 4 mg/day (Figure 2-3 a). Three of these seven participants reported differences between the two tools greater than the 95% LOA. A small number of outlier differences were also present in the UK cohort. Three UK participants reported much higher intakes in the CWSs compared to the 24DRs with differences of 5.59 mg/day, 6.16 mg/day, and 11.96 mg/day.

Table 2-5 Agreement of mean daily lutein and zeaxanthin intake between the monthly diet screener, combined weekly screeners, and multiple combined 24-hour diet recalls determined by Bland-Altman plot analysis in Australian and UK healthy adults.

Tool comparison		Mean difference [h]	Lower 95% LOA [h]	Higher 95% LOA [h]	R	R^2
AU	MS2 v 24DR(8) (n = 31)[a]	0.33 (0.00 – 0.67)	-2.91 (-3.24 – -2.58)	3.58 (3.24 – 3.91)	0.58*	0.35
	CWS(4) v 24DR(8) (n = 28)[b]	0.51 (0.00 – 1.03)	-1.46 (-1.97 – -0.95)	2.49 (1.97 – 3.00)	0.70*	0.67
	MS2 v CWS (4) (n = 34)[c]	-0.48 (-0.95 – 0.00)	-2.4 (-2.88 – -1.93)	1.45 (0.98 – 1.93)	0.83*	0.75
	MS1 v MS2 (n = 42)[d]	0.65 (0.00 – 1.3)	-3.21 (-3.86 – -2.56)	4.51 (3.86 – 5.17)	0.81*	0.59
UK	CWS(3) v 24DR(6) (n = 23) ^ [f]	1.32 (1.00 – 1.74)	0.37 (0.28 –0.49)	4.64 (3.52 – 6.11)	0.62**	0.12
CC	CWS(4) v 24DR(8) (n = 35) ^ [g]	1.23 (1.00 – 1.51)	0.57 (0.46 – 0.69)	2.66 (2.17 – 3.27)	0.75*	0.57

. Abbreviations: 24DR, 24-hour diet recall; AU, Australia; CC, combined cohorts; CI, confidence interval; CWS, combined weekly screeners; MS1,monthly screener 1; df, degrees of freedom; LOA, limits of agreement; MS2, monthly screener 2; SEM, standard error of the mean;(4) mean intake per day from the 4 weekly screeners,(8) mean intake per day from the eight 24-hour diet recalls,(3) mean intake per day from 3 or more weekly screeners,(6) mean intake per day from 6 or more 24-hour diet recalls. [a] AU MS2 vs 24DR(8) : SEM = 0.30, t value (30 df) = 1.12. [b] AU CWS(4) vs 24DR(8) : SEM = 0.19, t value (27 df) = 2.70. [c] AU MS2 vs CWS(4) : SEM = 4.7, t value (33 df) = –2.8. [d] AU MS1 vs MS2: SEM = 8.5, t value (41 df) = 2.1. [f] UK CWS(3) vs 24DR(6) : SEM = 0.06, t value (22 df) = 2.06. [g] CC CWS(4) and 24DR(8) : SEM = 0.03, t value (38 df) = 3.07. [h] Data presented as mg/day (95% CI). ^ Bland-Altman plot analysis values back transformed after Log10 transformation.* P < .001. ** P = .002.

The MS in the Australian cohort indicated a high test-retest reliability with a Cronbach's $\alpha = 0.86$ and two-way mixed effects model absolute intraclass correlation coefficient of 0.85. Despite being highly correlated, when divided into tertiles there was differences in classification of at least 30% in either direction between all tools (see Appendix B-3).

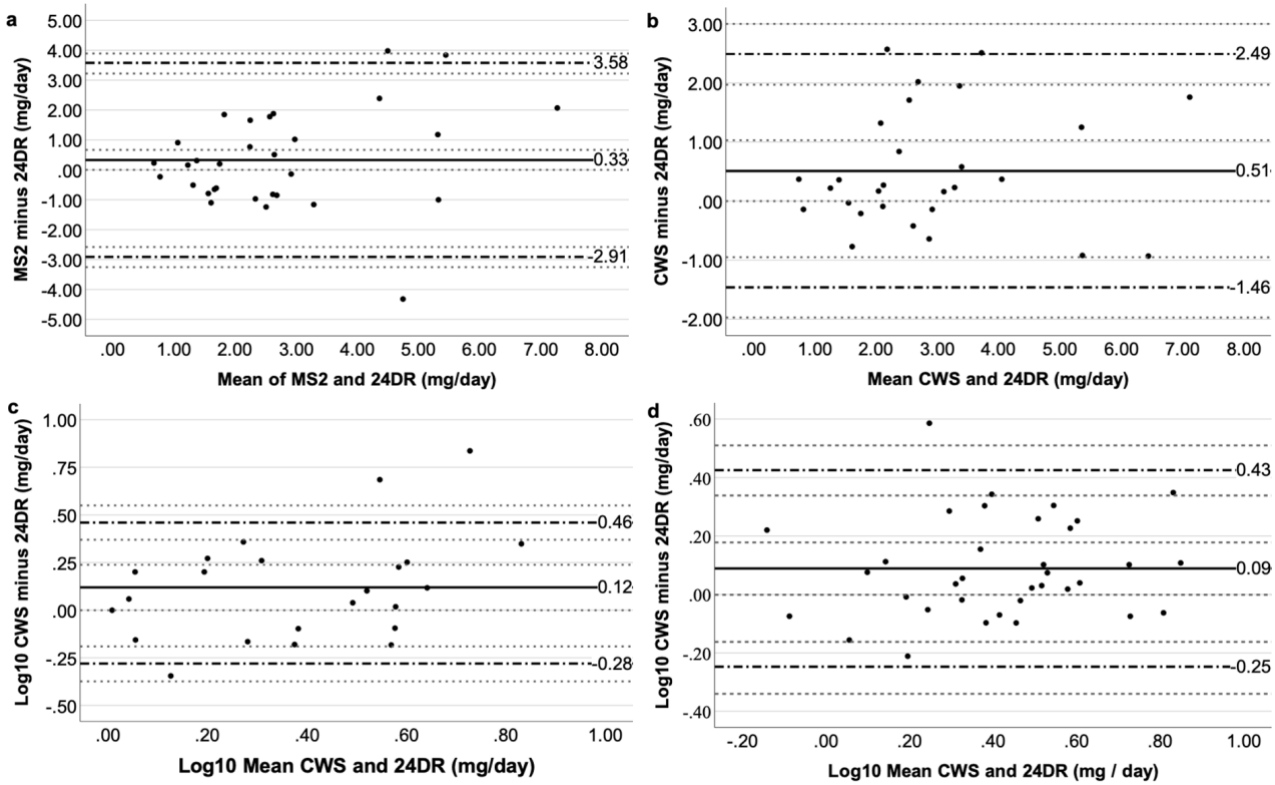

Figure 2-3 Bland-Altman plot analyses demonstrating poor agreement of daily dietary lutein and zeaxanthin intake between the monthly screener, combined weekly screeners, and multiple combined 24-hour diet recalls.
(A) Australian second monthly screener versus 8 combined 24-diet recalls. (B) Australian 4 combined weekly screeners versus 8 combined 24-hour diet recalls. (C) United Kingdom log base 10 transformed 3 or more combined weekly screeners versus 6 or more combined 24-hour diet recalls. (D) Combined cohort log base 10 transformed 4 combined weekly screeners versus eight 24-hour diet recalls. For each figure, black solid line indicates the mean difference, the black dashed lines indicate the 95% limits of agreement, and the grey dashed and dotted lines indicate the 95% confidence intervals for mean difference and 95% limits of agreement. Abbreviations: 24DR, 24-hour diet recalls; CWS, combined weekly screeners; Log10, logarithmic base 10; MS2, second monthly screener.

The contribution to L/Z intake from all food groups was consistent between the two screeners and cohorts (Table 2-6). The vegetable food group contributed the most to total L/Z dietary intake, with the contribution ranging from 87% to 91%. Fruits and the meat and alternatives groups were the next highest contributing sources, contributing between 3% and 6% to total L/Z intake.

Table 2-6 Percentage contribution to total lutein and zeaxanthin intake by the 6 food groups from the monthly diet screeners and combined weekly diet screeners in Australian and UK healthy adults.

Food Group	Australia			United Kingdom		Combined	
	MS1	MS2	CWS [4]	MS1	CWS [3]	MS1	CWS[4]
Vegetables	89.7 (80.7 – 93.0) [a]	91.2 (85.5 – 92.4) [a]	89.2 (80.9 – 92.3) [a]	88.0 (80.7 – 91.3) [a]	87.1 (82.1 – 92.6) [a]	88.3 (81.0 – 92.3) [a]	87.1 (82.2 – 91.9) [a]
Fruits	3.1 (1.1 – 7.0) [b]	3.5 (1.7 – 5.6) [b]	3.4 (2.1 – 5.2) [b]	5.7 (2.0 – 10.1) [b]	5.0 (2.5 – 8.5) [b]	4.1 (1.5 – 8.8) [b]	3.8 (2.3 – 7.1) [b]
Grains	1.6 (0.8 – 3.2) [b]	1.5 (0.9 – 2.8) [b]	2.0 (1.1 – 3.3) [b]	1.8 (1.1 – 2.9) [c]	2.4 ± 1.5[c]	1.7 (0.9 – 2.9) [c]	2.0 (1.2 – 3.1) [c]
Meat and alternatives	3.2 (1.8 – 6.1) [b]	3.9 (1.9 – 6.0) [b]	4.5 (2.7 – 7.9) [b]	3.3 (1.6 – 6.0) [b]	3.5 (1.8 – 7.1) [b]	3.3 (1.8 – 6.0) [b]	4.6 (2.7 – 7.8) [b]
Milk, yoghurt, cheese, and alternatives	0.3 (0.1 – 0.6) [c]	0.3 (0.1 – 0.5) [c]	0.3 (0.2 – 0.7) [c]	0.3 (0.0 – 0.5) [d]	0.3 (0.1 – 0.8) [d]	0.3 (0.1 – 0.6) [d]	0.3 (0.2 – 0.7) [d]
Discretionary foods	0.3 (0.2 – 0.6) [c]	0.3 (0.2 – 0.6) [c]	0.4 (0.3 – 0.7) [c, 1]	0.2 (0.1 – 0.4) [d, 1, 2]	0.4 (0.1 – 0.7) [d]	0.3 (0.1 – 0.4) [d]	0.4 (0.3 – 0.7) [d, 2]

Abbreviations: CWS, combined weekly screeners; L/Z, lutein and zeaxanthin; MS1, monthly screener 1; MS2, monthly screener 2; [4] 4 combined weekly screeners; [3] 3 or more combined weekly screeners. Data presented as median (25th–75th percentile) or mean ± standard deviation percentage (%) contribution to total L/Z intake. [a, b, c, d] Within a column, cells with the same superscript letter were not significantly different to each other. [1,2] Indicate within a row a significant difference between tools with the same number.

The foods that contributed the most to total L/Z intake were similar between the Australian and UK cohorts (Table 2-7). In the Australian cohort, baby spinach contributed the most with between 13% and 22% to total L/Z intake across the screeners. Additionally, baby spinach, cooked pumpkin and cooked broccoli combined made up approximately a quarter (23% – 31%) of total L/Z intake across the screeners. Other contributing foods included cooked zucchini, carrot, lettuce and cooked egg. In the UK cohort the major contribution was more evenly distributed between six foods, with cooked broccoli, cooked green peas, baby spinach and lettuce combined contributing 19% to 22% of total L/Z intake across the screeners. Other high contribution foods included cooked egg, and cooked and raw orange carrot.

Table 2-7 Top 6 ranked foods in percentage contribution to total lutein and zeaxanthin intake from the monthly diet screeners and combined weekly screeners in Australian and UK healthy adults.

Tool			1st	2nd	3rd	4th	5th	6th
AU	MS1	Food	B. spinach [b]	Broccoli	Pumpkin	Zucchini	O. carrot [b]	Lettuce [b c]
		%	17.6 (2.8 – 26.8)	5.4 (2.1 – 8.8)	4.2 (0.0 – 10.9)	4.0 (0.0 – 8.1)	2.6 (0.0 – 7.0)	2.6 (0.0 – 6.1)
	MS2	Food	B. spinach [b]	Pumpkin	Broccoli	O. carrot [b]	Zucchini	Lettuce [b c]
		%	21.9 (0.0 – 30.1)	5.0 (0.0 – 10.2)	4.5 (1.7 – 9.1)	2.9 (0.0 – 9.3)	2.7 (0.0 – 5.4)	2.6 (0.0 – 6.1)
	CWS	Food	B. spinach [b]	Pumpkin	Broccoli	Egg	Lettuce [b c]	Zucchini
		%	13.6 (5.6 – 35.3)	5.8 (0.0 – 12.1)	4.2 (0.7 – 8.9)	3.1 (1.2 – 4.8)	2.6 (0.3 – 6.1)	2.5 (0.0 – 7.0)
UK	MS1	Food	Broccoli	Green peas	B. spinach [b]	Lettuce [b c]	O. carrot	Egg
		%	6.8 (3.2 – 12.4)	5.0 (2.5 – 10.5)	3.7 (0.0 – 5.8)	3.4 (0.0 – 9.3)	2.6 (1.2 – 4.9)	2.6 (0.9 – 4.6)
	CWS	Food	Broccoli	Green peas	Lettuce [b c]	O. carrot [b]	B. spinach	O. carrot
		%	7.7 (4.1 – 9.9)	5.7 (1.4 – 14.7)	5.3 (2.4 – 10.2)	5.0 (0.8 – 7.7)	3.6 (0.0 – 10.1)	2.7 (1.6 – 5.9)
Cb	MS1	Food	B. spinach [b]	Broccoli	Green peas	O. carrot [b]	O. carrot	Egg
		%	14.0 (0.00 – 22.2)	7.8 (2.8 – 1.4)	3.8 (0.0 – 6.7)	2.6 (0.0 – 5.9)	2.2 (0.4 – 3.6)	2.1 (1.0 – 4.5)
	CWS	Food	B. spinach [b]	Broccoli	Lettuce [b c]	Egg	O. carrot [b]	B. spinach
		%	8.1 (0.0 – 22.6)	5.8 (2.4 – 9.4)	3.2 (1.5 – 7.1)	3.0 (1.3 – 4.9)	2.4 (0.0 – 7.0)	2.3 (0.00 – 9.9)

Abbreviations: B, baby; CC, combined; CWS, combined weekly screeners; MS1, monthly screener 1, MS2, monthly screener 2; O, orange. Data presented as median (25th–75th percentile). [b] Indicates a raw food, all other foods in cooked form. [c] Type of lettuce Cos or Romaine.

2.6 Discussion

Intakes reported between the screeners and 24DRs indicated poor agreement via Bland-Altman plot analysis but significant moderate correlations (Table 2-5). The 95% LOA of the MS and CWSs compared with the 24DRs were at minimum greater than 0.25 mg/day, therefore indicating that the screeners were not valid in the population observed. [33, 67] The WS agreed best with the 24DRs, reporting a mean difference of 0.51 mg/day and 95% LOA between -1.46 and 2.49 mg/day in the Australian cohort. There was no clear trend in the direction of differences reported between any of the tools. The mean differences between the tools were trending toward the screeners reporting higher L/Z intakes compared with the 24DRs. This aligns with similar studies comparing an FFQ or screener intake against 24DRs or diet records outlined below. [152, 153] The median dietary L/Z

intake of the combined cohorts was between 2.4 and 3.4 mg/day (Table 2-4). This intake aligns with mean intakes of 0.5 to 4.5 mg/day measured by FFQ in previous Western country populations. [13, 97, 166, 167]

The MS and WS had poor validity for ranking participants by intake. High misclassification rates of 38% to adjacent tertiles were observed with the CWSs when ranked by the MS2 (Appendix B-3). The inability to rank participants into tertiles between MS2 and CWSs indicates that these two screeners cannot be used interchangeably. Logarithmic base 10 transformation and reliability testing of the MS1 and MS2 data resulted in a normal data distribution, a Cronbach's alpha of 0.88, and absolute intraclass correlation coefficient of 0.78. Despite a high absolute intraclass correlation coefficient, the 31% misclassification observed between the MS1 and MS2 was higher than previous similar validation research [153]. In the validation study by Satia et al.[153], a FFQ with a recall timeframe of a month ranked participants intakes into quartiles. Of all antioxidant nutrients investigated, the range of classification into the same or adjacent quartile was between 65% and 89%, and only 0% to 12% misclassification into the opposite quartile. [153] Exact rates of misclassification for L/Z were not reported. The multi-directional high misclassification of the MS and WS observed in the present study indicates the screeners were not able to rank participants consistently by intake and are thus not valid for ranking participants in intervention or observational study designs.

Previous validation studies have returned poor tool validity when attempting to capture total dietary or antioxidant intake, sometimes inclusive of L/Z. [143, 151-154] Comparison with prior studies is difficult due to the frequent use of correlation statistics rather than assessing agreement through a Bland-Altman plot analysis. Similar to the present study, prior research has often relied upon the USDA FCT to calculate L/Z dietary intake. [152, 153] A study in 28 Australian adults compared a FFQ with a 6-month recall timeframe with 12 days of diet records completed over one year. Mean ± SD daily L/Z intake reported from the diet records and FFQ were 0.52 ± 0.26 mg and 1.63 ± 1.17 mg respectively. The reported intakes were significantly correlated, with a correlation coefficient of 0.40 ($p < 0.05$). Plasma L/Z was also measured and used to report a validity coefficient calculated by the method of triads. The low validity coefficient (95% CI) for L/Z of 0.19 (0.05 – 0.71) indicated that the FFQ did not provide a valid measure of L/Z intake. [152] The small sample size and misaligned timeframes of dietary data collection were proposed as explanations for the poor validity. The diet records were completed after the FFQ and plasma measurement. In the present study, the timeframes of dietary data collection were more closely aligned with the WS and MS. Participants were asked to recall intake over the same timeframe during which the 24DRs were collected. This closer alignment is reflected in the higher correlation coefficients of 0.58 ($p < 0.001$) between the MS and 24DRs and 0.70 ($p < 0.001$) between the CWSs and 24DRs in the Australian

cohort. Another study that utilized closely aligned recall timeframes was conducted in 81 white and 83 African American adults. It compared the data from a FFQ with a recall timeframe of a month against four telephone administered 24DRs. Two of the 24DRs were completed on a weekday and two on weekend days in the month preceding the FFQ. Median (25^{th} – 75^{th} percentile) daily L/Z intake reported by the FFQ was 3.03 (1.61 – 4.84) mg for white participants and 1.94 (1.06 – 3.98) mg for African American participants. Median (25^{th} –75^{th} percentile) daily L/Z intake reported by the 24DRs was 2.41 (1.20 – 3.69) for white participants and 1.63 (0.93 – 2.91) for African American participants. The significant adjusted correlation coefficient between the two tools was 0.49 for white participants and 0.51 for African American participants, $p \leq 0.0001$. [153] Intake representative of a month may have been difficult to capture with just four 24DRs due to inter-day intake variability. [69] In the present study, the large number of 24DR days captured may explain the stronger correlations observed between tools. The Australian and combined cohorts CWSs and 24DRs comparison indicated correlation coefficients of 0.70 ($R^2 = 0.67$) and 0.75 ($R^2 = 0.57$) respectively. The moderate correlation but poor Bland-Altman agreement observed raises concerns regarding the utility of results obtained in prior L/Z validation studies reliant on correlational statistics. The linear relationship between two dietary intake tools measuring the same component as demonstrated by correlation statistics is arguably not enough to demonstrate validity. [164] Unlike a Bland-Altman plot, correlation statistics do not provide an indication of the bias between tool differences or an indication as to what degree of difference is appropriate. [165] As demonstrated in this study, the MS and CWSs were both moderately correlated with the 24DRs. However the Bland-Altman plot demonstrated the poor agreement, reasons for that poor agreement, and therefore the tools' invalidity. Without the use of a Bland-Altman plot, correlation statistics would have overestimated the validity of the MS and WS. Prior L/Z or antioxidant questionnaire validation studies, solely reliant on correlational statistics to determine validity, should be interpreted with caution. The absence of a validated tool to capture habitual dietary L/Z intake remains a barrier to understanding the diet-disease and dose-response relationships between dietary L/Z intake and conditions such as age-related macular degeneration. It also precludes identifying a daily dietary intake recommendation for L/Z. [67]

The poor Bland-Altman agreement and the screeners' inability to rank participants by intake compared to the 24DRs may be explained by misestimation or missed capture of a small subset of foods such as those listed in Table 2-7. Misestimation refers to the incorrect recall of the amount or frequency of intake of a food. Missed capture refers to true intake of a food not being captured due to the timeframe being observed through a particular tool. The misestimation or missed capture of foods may partially explain the emerging trends of higher L/Z intakes being reported through FFQ or screener tools compared to 24DR or diet record tools. Some of these foods, including baby

spinach, are high L/Z concentration foods that are sporadically consumed in amounts difficult to estimate by volume or weight. The misestimation or missed capture of such foods was particularly obvious in participants reporting high consumption of L/Z. Seven Australian participants reported a combined MS2 and 24DR mean daily L/Z intake greater than 4 mg/day and were more likely to report larger differences in intake between the MS2 and 24DR. Three of these seven participants reported differences between the two tools greater than the 95% LOA (Figure 2-3a). These larger differences occurred through poor agreement in reported vegetable consumption, particularly green leafy vegetables. For example, the participant with a difference of -4.32 mg/day between the MS and 24DRs reported that 90% of L/Z intake was from vegetables in the MS2. The top three foods being 34.6% from cooked frozen baby spinach, 16.5% from cooked kale, and 14.9% from raw baby spinach. Similarly, three UK participants reported high L/Z intake and large differences between the CWSs and 24DRs. The differences in L/Z intake between the CWSs and 24DRs for these three participants were 5.59 mg/day, 6.16 mg/days, and 11.96 mg/day. These differences related to green leafy vegetables (kale, baby spinach, rocket, silver beet), broccoli, green pea and carrot intake. More representative capture of these high contribution foods is needed in future validation attempts. Understanding how errors have occurred is necessary to improve how intake is captured more accurately from these vegetables. Differences may have occurred through repeat errors with moderate concentration foods such as carrot (0.3 mg cooked and 0.7 mg raw of L/Z per 100 g food), or infrequent errors with high concentration foods such as baby spinach (>0.6 mg/100g L/Z). [138] The impact of misestimating intake of a high concentration food such as baby spinach can be observed in one participant's reported WS and MS intake. Across the four WSs completed, this participant reported five serves of baby spinach, equaling a total of 13.3 mg of L/Z for the month. Comparatively, in the MS2 this participant only reported four serves of baby spinach; a total of 10.6 mg L/Z and difference of 2.7 mg (or 0.1 mg/day) to the CWSs. The difference in baby spinach intakes reported between the CWSs and MS2 demonstrates the impact of memory recall bias and difficulty in estimating food volumes [75, 76]. In particular, green leafy vegetables appear to pose an issue. Their inclusion in mixed dishes, their light but voluminous nature in raw state and stark volume shrinkage when cooked, make it difficult for participants to estimate intake weight or volume in metric cups. To improve the validity of the MS and WS, the inclusion of a photographic atlas with real size food portions, including portion in multi-ingredient dishes, to visually assist participants when estimating food intake is justified. [91, 97, 104]

Missed capture of impactful foods such as baby spinach, must also be addressed to improve the validity of the screeners. The aforementioned participant who reported four or five baby spinach serves over the month also demonstrated a likely example of missed capture. The total number of baby spinach serves reported from all eight 24DRs combined was only 1.75 serves. The 24DRs may

have underestimated mean L/Z intake over the month. In this case, poor agreement between the 24DRs and screeners occurred due to the presence of an irregularly consumed food and utilization of a dietary intake method that did not adequately capture habitual intake. [68, 75] It appears eight non-consecutive days of 24DRs over four weeks was insufficient to capture inter-day variation in dietary L/Z intake. Missed capture of L/Z intake by the 24DRs may mean the validity of screeners has been underestimated, and that the screeners may actually be better at capturing habitual dietary L/Z intake than the Bland-Altman plot analysis suggested. Future studies would be strengthened with the addition of a biological marker such as blood L/Z concentration to correlate with the L/Z intake captured by the screeners. [152] Additionally, the dietary intake method selected to perform relative validity against the screeners should consider the impact of missed capture observed in this study. Future studies should consider balancing participant burden, and the benefit from capturing greater number of days of dietary intake through 24DRs or alternative methods such as non-consecutive repeated 3-day diet records. [71]

The limitations in this study include the origin of the FCT used for intake analysis, the substantial missing data and attrition rates, low demographic diversity of the cohort, and the lack of a biological marker. The use of the USDA FCT is a limitation to determining accurate intake, because the data on food composition likely differs to the local food supply of the study cohorts. It may differ for many reasons, including variation in plant cultivar, growing and food storage conditions, and extraction and analysis methods to determine concentrations. [168-170] The use of the USDA FCT was unavoidable in this study due to the paucity of data about L/Z concentrations in the Australian and UK food composition databases. [138, 158, 171] Using local data is critical to accurately represent intake. For example, cooked green peas have a L/Z value of 2590 μg/100 g in the USDA FCT (identification 170420), 1134 μg/100g in the UK McCance and Widdowson's dataset (food code 13-527), and 620 μg/100 g in the Australian FSANZ table (identification F006538). The missing data and high attrition rates limited the strength of validity testing across all tools and cohorts. The goal of 30 or more participants per Australian and UK cohort was only achieved for the comparison of the MS2 against the 24DR and MS1 against the MS2 in the Australian cohort. The high study burden was reported as a reason for attrition. Additionally, the predominantly female and tertiary-educated characteristics of participants who did complete the study are not generalizable to the overall Australian or UK population. Finally, measuring concomitantly blood L/Z concentration as a biological marker was not considered due to COVID-19 pandemic restrictions at the time of data collection. Future research to validate the screeners should aim to capture a more diverse population and include a biological marker of L/Z intake to allow for the triad method of validation. To reduce participant burden, the use of less intensive

dietary intake collection tools spaced out over a longer timeframe such as 6, 12 or 24 months could be considered. The longer timeframe would also allow for greater likelihood of capturing habitual intake, as L/Z containing foods were observed to be episodically consumed in this study, and consumption may change seasonally across the year.

2.7 Conclusion

A valid tool to capture habitual dietary L/Z intake is important to progressing the understanding of the diet-disease and dose-response relationships between dietary L/Z intake and conditions such as age-related macular degeneration. [67] These L/Z specific screeners were not valid, demonstrating poor agreement and ability to rank participants according to intake compared with L/Z intake derived from multiple 24DRs. Dietary L/Z intake between the screeners and 24DRs for the Australian and UK cohorts both individually and combined were moderately to strongly correlated. Despite significant correlations, the Bland-Altman plots indicated that participants were unable to accurately recall intake of L/Z containing foods, particularly green leafy vegetables. The phenomenon of strong correlation but poor Bland-Altman plot agreement observed in this study suggests that results from prior research reliant only on correlation statistics must be interpreted with caution. Only a small number of foods, such as baby spinach and broccoli, contributed markedly to dietary L/Z intake in this study. Accurate representation of these high contribution foods in local FCT and capture of intake through screeners should be the focus of future validation attempts. In addition, to improve the validity of the screeners, future studies would benefit from a larger, more diverse study sample, a lower participant burden study design to reduce attrition rates, the addition of a photographic atlas to assist with accurate food volume estimation, the use of a local FCT data, and the use of a concomitant biological marker.

2.8 Summary

This chapter directly addresses the component of the overall thesis aim of exploring a method to quantitatively measure habitual dietary L/Z intake. The MS and WS were developed to address the primary literature gap identified from the narrative review in section 1.3, identified as barrier 1 in Figure 1-3 (page 61). Additionally, the screeners were developed to address thesis objective 1, the development and validation of a dietary screener designed to capture habitual dietary L/Z intake. The screeners were successfully developed but the validation process revealed poor validity; thus this thesis objective was only partially met. Healthy adults were unable to report comparable dietary L/Z intake through dietary screener compared with multiple 24-hour diet recalls. In relation to the nine criteria, a method to quantitatively measure habitual dietary L/Z is needed to conduct cohort

and dose-response studies which relate to criteria 4 and 6 respectively (Figure 2-4). The finding that FCTs values may be impacting the validity of a dietary screener relates to criterion 3.

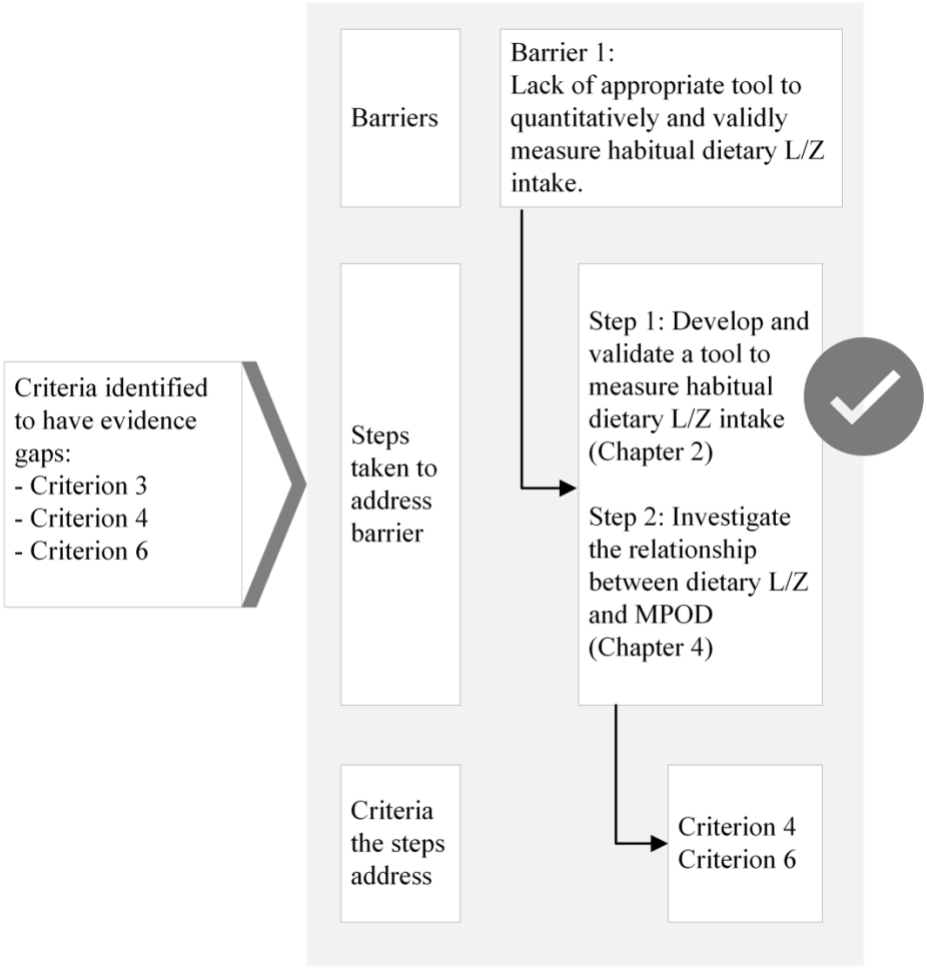

Figure 2-4 Steps addressed as part of Chapter 2 to improve the lutein and zeaxanthin evidence base related to the 9-criteria by Lupton et al. [2]

Chapter 3 Assessing electronic device use behaviours in healthy adults: development and evaluation of a novel tool

This chapter reviews literature relevant to the role of electronic devices (ED) in macular health and macula L/Z concentrations (section 3.1). The literature explores factors that I considered in the development of the new ED use tool. These factors include, sources of blue light (BL) exposure, implications of BL from EDs on the macula, measuring macular L/Z concentrations, and options for a new tool to monitor ED use. Additionally, this chapter describes my original research study addressing thesis objective 2 (section 3.2 – 3.8), the development and validation of a questionnaire to capture usual ED use behaviours in Australian and UK adults.

3.1 Reviewing the implications of electronic device use on macula lutein and zeaxanthin concentrations

The plausible biological rationale (Figure 1-1, criteria 9, page 31) for L/Z to have a dietary intake target is most strong with the established link between macular L/Z and lifetime macular function and disease prevention, such as AMD. [1, 10] The measurement of MPOD as an estimation of macular L/Z concentrations has been investigated as a proxy marker for AMD risk for over 20 years. [32] In order to establish a dietary L/Z intake recommendation that positively impacts MPOD, and subsequent risk of AMD, the relationship between dietary intake measured with a valid tool and MPOD must be established. As identified earlier (section 1.3.5), the dose-response relationship between dietary L/Z interventions and MPOD is presently unclear. In part it is unclear due to lack of valid dietary intake tools to capture and explore the impacts of habitual L/Z intake. [67] An additional reason the dose-response relationship may be unclear is the confounding of the relationship by BL exposure from EDs. Blue light exposure from EDs is an emerging environmental exposure that may negatively impact macular L/Z concentrations and macular health. [10, 172]

An understanding of the dose-response relationship between L/Z intake and MPOD is important as it relates to criterion 6, clinical trials for dose-response and efficacy (Figure 1-1, page 31). Therefore, to effectively explore the relationship between MPOD and dietary L/Z intake the potential role of ED use as a confounder in this relationship must be understood (Chapter 4). Before this relationship can be investigated, a tool to capture ED use is needed (section 3.3 – 3.8).

3.1.1 Blue light exposure and macular health

Blue light from electronic devices (ED) is an emerging confounding factor when investigating L/Z macular concentrations. It may be a confounding factor due to hypothesised macular damage as a

result of BL exposure. It has been proposed that BL exposure has the potential to increase oxidative stress in the macula. A function of L/Z is prevention of ROS production through BL absorption and direct antioxidant activity to manage oxidative stress. [10, 172] It is plausible that the interaction of L/Z with BL exposure, such as from EDs, may contribute to fluctuations in L/Z macular concentrations. Thus, BL exposure from EDs is an emerging factor to consider when investigating the relationship between dietary L/Z intake and MPOD.

3.1.1.1 Blue light

Blue light is part of the electromagnetic spectrum. Electromagnetic radiation includes ultraviolet radiation (UVR) that is 100 – 400 nm, visible radiation that is 400 – 760 nm, and infrared radiation (IR) that is 760 – 10,000 nm. [23, 173] Visible light is referred to as either blue (short wavelength), green (medium wavelength) and red (long wavelength) light. Shorter wavelengths of light are higher energy compared to longer wavelengths. These high energy photons therefore have greater potential to excite molecules irradiated by them.

3.1.1.2 Sources of blue light

The sun is the most potent source of light humans are regularly exposed to. It emits a broad spectrum of electromagnetic radiation from ultraviolet through to short infrared wavelengths. BL is also emitted by artificial lighting such as household or street lighting, and ED screens. Technological advancements have increased human exposure to artificial sources of high energy BL. The development of the light emitting diode (LED) has meant longer lasting and energy efficient light sources. However, the emission spectra of LEDs are predominantly blue, compared to other light sources such as incandescent light globes. It is currently unknown whether chronic exposure to LEDs may be increasing the risk for photochemical damage on the retina. [172, 174] LEDs are increasingly being used for general lighting purposes, and as the lighting technology for ED screens. With the current integration of technology and daily life activities, it has created an environment in which humans are regularly exposed to high energy wavelengths of light from an early age for extended periods of time.

3.1.1.3 Visible light radiation guidelines and recommendations

Guidelines surrounding ocular radiation exposure are influenced by location of interest in the eye and light exposure wavelength and quantity. Radiation below 295 nm, UVR, is absorbed predominantly by the cornea of the eye. While UVR between 280 – 400 nm, known as UV-B and UV-A, are absorbed by the lens. The lens is not completely efficient and thus a small fraction of

UV-A is transmitted to the retina. The visible light spectrum (400-760nm) is transmitted to the retina, stimulating the photoreceptors and initiating the visual process. With age, the opacity of the lens increases, and less short wavelength light is transmitted to the retina. [23, 175]

The International Commission on Non-Ionizing Radiation Protection (ICNIRP) guidelines defines several radiometric quantities used in measuring light exposure. [176] Radiometry is measurement of radiant energy, including light, in terms of absolute power. The radiometric quantities are power (W), energy (J), irradiance (W m^{-2}), radiant exposure (J m^{-2}), radiance (W m^{-2} sr^{-1}), and radiance dose (J m^{-2} sr^{-1}). Radiant exposure is the quantity of exposure, or dose, and irradiance is the dose-rate. Radiance and radiance dose integrate time of exposure. [176] These measures have a photometric analogue. Photometry addresses how these measures of light are perceived by the human eye and looks only at the visible light spectrum. The photometric quantities include luminous flux (lumen, lm), luminous intensity (lm sr^{-1}), illuminance (lm m^{-2} or lux), and luminance (cd m^{-2}). A lumen is the quantity of energy emitted into unit solid angle (1 sr) by an isotropic point source having a luminous intensity of 1 candela (cd). Illuminance is irradiance spectrally weighted with the photopic human eye sensitivity curve. Luminance is the luminous intensity per unit area of light travelling in a given direction, describing light passing through, emitted or reflected from a particular area falling within a given solid angle. [177]

The ICNIRP guidelines were published in 2013. Guidelines to avoid retinal toxicity from BL exposure were proposed for acute exposures (less than eight consecutive hours) and do not consider chronic exposure. [176] In an acute setting, radiance from ED screens is reported to be less than 10% of the ICNIRP BL photochemical exposure limit. The ICNIRP exposure limit for approximately 3 hours is 100 W m^2 s^{-1}. [177] However, exposure to BL from LED or organic LED sources occurs for extended periods of time each day, and potential effects from this chronic exposure remain unknown. Committees of experts with reported position statements on BL exposure include ICNIRP, Scientific Committee on Health, Environmental and Emerging Risks, Agency for Food, Environmental and Occupational Health and Safety, Federation of National Manufacturers Associations for Luminaires and Electrotechnical components (European Union), and European Lamp Companies Federation. These committees conclude there is currently no known long-term risks to BL exposure from LEDs. The committees also indicated that continued monitoring and research into potential damage from LED BL exposure is warranted. [176-178] Whilst not cause for alarm, continued investigation is warranted due to the mechanistic plausibility for retinal damage supported by animal studies. [179] In addition, the LED form of BL exposure is a new phenomenon with no longitudinal data on the long-term exposure effects available.

3.1.1.4 Mechanism for electronic device related retinal damage and implications

There are three proposed mechanisms by which light may cause damage within the retina: photomechanical, photothermal and photochemical. The focus here is on photochemical damage as it is a mechanism of damage relating to BL. Photochemical damage is retinal tissue injury from exposure to ROS that have been generated as a result of light exposure. The two factors that influence photochemical damage are the duration and the wavelength of light exposure. Within the retina there are photosensitive regions in molecules known as chromophores. Examples of chromophores include photoreceptors, lipofuscin, and melanosomes. A chromophore is a region in a molecule that has an absorbance within the visible light spectrum. That is, the energy difference between ground state and excited state of electrons falls between 400 – 760 nm. An excited chromophore may return to ground state through reemission of a longer wavelength of radiation or heat dissipation. [172, 180] However, chromophores may also generate ROS through splitting the bond in another molecule via direct electron or hydrogen exchange, or transfer energy to oxygen resulting in singlet oxygen species. [23, 181] The blue component of the visible light spectrum is higher in energy and therefore has greater potential to cause photochemical damage at the retina. Photoreceptors within the eye initiate the visual transduction process. This process is energy consuming and has a high oxygen demand. To facilitate this process, photoreceptors have extremely high concentrations of mitochondria. Additionally, fatty acid DHA is highly concentrated in the membrane of photoreceptor outer segments and is known to be susceptible to peroxidation from ROS. Therefore, this oxygen rich tissue that is exposed to high energy radiation constitutes an environment susceptible to the generation of ROS and the resulting damage. [182]

Exposure to BL has been shown to cause photochemical damage to the retina in both in vitro and animal studies. [179, 183-185] A study in 6-week old Winstar rats showed disruption of retinal pigment epithelium tight junctions after 6 hours of LED exposure, retinal radiant exposure of 5.23 J / cm^2. With 18 hours of LED exposure, retinal radiant exposure of 15.7 J/cm^2, serum albumin was found to have leaked in the interphotoreceptor space. Retinal radiant exposure is a calculation of the light reaching the retina when accounting for the source of light, environment, and spectral transmittance of rat ocular media. [179] The retinal pigment epithelium is a single cell layer, containing pigmented granules, that ensures function of photoreceptors for the visual transduction process. [186] Another study exposed Sprague-Dawley rats to either single wavelength blue LED (460 nm), white LED, white compact fluorescent lamp or yellow compact fluorescent lamp at 750 lux for 12 hours / day for either 3, 9 or 28 days. The results of the electroretinogram showed a significant decrease in photoreceptor function, measured by lower b-wave peak, compared to the control group at 9 and 28 days for rats exposed to the blue LED or the white LED, $p < 0.001$.

Electroretinography is a measure of the responsiveness of rods and cones. Dissected retinas showed a significant decrease in outer nuclear layer (anterior to photoreceptor layer) thickness with white and blue LED exposure by 9 days, $p<0.01$. [183]

In vitro studies using human retinal cell lines have also shown increased markers for damage with exposure to the BL. [184] Adult retinal pigment epithelial cell line (ARPE-19) cultures have been treated with the photosensitive component of lipofuscin and exposed to BL observe changes in oxidative stress. Lipofuscin is an autofluorescent lipid-protein aggregate that accumulates in retinal pigment epithelium cells over the lifetime. By 80 years of age, lipofuscin may accumulate to occupy 19% of cytoplasmic volume in retinal pigment epithelium cells. [187] Lipofuscin is formed due to the incomplete lysosomal degradation of photoreceptor outer segments that have been shed for renewal. [188, 189] A principal photosensitive component of lipofuscin is N-retinylidene-N-retinylethanolamine (A2E). The A2E component is a derivative of vitamin A from the visual transduction process. Irradiation of lipofuscin from visible light produces ROS, specifically superoxide anions, hydrogen peroxide, and singlet oxygen species. With age-dependent increase in lipofuscin, the potential for ROS production and subsequent oxidative damage increases. [189-192] In an in vitro study by Moon et al.[184] ARPE-19 cell cultures were treated with A2E and exposed to ED with three variations of BL peak wavelength. Three ED emitting only BL and three devices displaying a white image. The BL only devices displayed an intensity of 0.04 W $(m^2\ sr\ nm)^{-1}$. The white displays adjusted the intensity of blue, red and green light for each BL wavelength to ensure a consistent luminance of 500 cd cm^{-2}. The device technology and peak BL wavelength were a liquid crystal display with a BL peak at 449 nm, and organic LEDs at 458 nm or 470 nm. An ARPE-19 culture treated with A2E and incubated in darkness was used as a control. Results from BL only devices showed that ROS production increased in a time dependent manner. After 24 and 48 hours of 449 nm or 458 nm BL exposure, ROS production was significantly increased compared to the control culture. Cell viability was significantly decreased compared to control across all three BL only wavelength groups; the marker used to assess cell apoptosis, caspase-3/7, increased significantly compared to the control. Results from exposure to white displays also indicated significantly decreased cell viability and increased caspase-3/7. Despite this being an in vitro study, it indicates a plausible mechanism of retinal damage from low intensity display devices. [184] Notably, the luminance of the white displays used (500 cd cm^{-2}) were selected to imitate that of every day smartphones. Luminance of typical screen content from recent smartphone displays have been reported to range between approximately 350-750 cd cm^{-2}. Comparatively, luminance of newer organic LED televisions is reported at 540 cd m^{-2} or more. [193]

Both the public, health professionals and researchers are aware of the potential damage from BL, however the consensus surrounding whether humans should be attempting to reduce BL exposure and/or use protective devices is not agreed upon. [174, 194] An Australian cross-sectional study surveying optometrists' opinion toward BL blocking lenses showed 75.3% of respondents prescribe BL blocking spectacle glasses in their practice. Additionally, approximately 44% of optometrists felt daily environmental BL exposure is a potential cause of retinal damage, and 50% felt placebo effects may play a role in patient's responses to use of BL blocking lenses. [195] The BL blocking glasses, and intra-ocular lenses do not filter enough BL to be consisted personal protective equipment (in regards to reducing risk of acute retinal phototoxicity). A recent trend to reduce BL exposure is changing ED colour temperatures to reduce the quantity of BL in the device spectrum emitted (changing the ED image to a warm white). [178] This change is possible from technology brands such as f.lux on computers and night-time on Apple Inc. handheld devices. [196] Technology is advancing quickly, and the potential repercussions are unknown. Relative to the depth of the research evidence base at present, the commercial distribution of information has been proposed to be misused to over alarm individuals in reference to potential damage from ED BL exposure. [194] It is important to note that that present evidence does not indicate that chronic exposure to BL from ED is damaging or increases risk of AMD. However, as the research has not been done, it is also not confirmed that chronic BL exposure from EDs is free from risk or harm. [174] The interest of BL exposure from EDs in this thesis is less about whether exposure is 'good' or 'bad', but whether this exposure is impacting MPOD. If exposure is negatively impacting MPOD it will influence how attempted measurement of dietary L/Z intake is able to be correlated to MPOD. In addition, if exposure is negatively impacting MPOD this may need to be considered in the development of a dietary target for L/Z.

3.1.1.5 Current measurement of electronic device use

To begin investigating whether BL exposure from ED is impacting MPOD, a method to capture human behaviours surrounding use must be available. Advancements in technology have seen EDs become essential components of modern-day society, particularly in developed countries. [197] EDs include display devices such as smartphones, tablets, computers, and televisions (TV). Reports on ED use to date have been through commercial entities using questionnaires or interviews with unknown validity. [198-200] The 2019 Deloitte mobile and media reports indicated that 9 of every 10 Australians own a smartphone they use on average 3 hours / day, and average daily television use is just over 3 hours. [198, 199] A UK commercial report, the UK based Ofcom 2018 Communications Market Report, also indicates that since 2008 ED ownership and use has increased. Smartphone ownership increased from 17% to 78%. Additionally, from self-reported

recall, daily average time spent online (activities involving internet use) inclusive of all EDs was more than 40 hours/week for 1 in 5 adults. [200] The prolonged and chronic exposure to EDs has been flagged as a potential issue not just in respect to macular damage, but also several other health-related issues such as digital eye strain (also known as computer vision syndrome), musculoskeletal disorders and sleep disturbances. [197, 201, 202] In 54 Australian adults postural habits with smartphone use was investigated and it was found that inter-day smartphone use was highly variable and mean participant use was 2.6 ± 1.5 hours/day (range: 0.5 – 7.4 hours/day). [202] The hours of daily smartphone use was accumulated over multiple sessions or 'phone uses', the mean number of sessions was 51.7 ± 34.9 per day and session length ranged from 1.0 seconds in length up to 3.4 hours. Smartphone use was captured for seven days by a phone app called RealizD (RealizD Pty Ltd) that records the users screen time whilst unlocked. Whilst this study was able to capture personal use of smartphone phones it was limited in that it did not capture all device use or use of shared devices. Use of EDs is increasing, and thus exposure to potentially damaging BL from these sources is also rising. Currently, there are no validated or specific tools for quantifying human exposure to BL from all EDs. To determine the ED impacts of BL on the macular a method to quantify acute and chronic exposure must be created.

3.1.1.6 Electronic device use in relation to macular lutein and zeaxanthin concentrations

To determine the potential negative implications of ED BL exposure on macular health in humans, ED use should be investigated in relation to a marker of macular health. As previously discussed, MPOD is an estimation of macular L/Z concentrations and has been utilised as a proxy for AMD risk for over 20 years. [32] One of the mechanisms by which BL exposure may be negatively impacting macular health is through photochemical damage. In response to this damage L/Z may be acting directly as an antioxidant therefore lowering macular L/Z concentrations. [23, 181] Thus, MPOD may be an appropriate marker to utilise in the investigation of ED use impacts and macular health.

Understanding how MPOD can be measured and the strengths and limitations of available methods is important to ensure effective investigation of any relationships between ED and MPOD. MPOD is estimated by subjective and/or objective methods. Subjective methods require close involvement and a response from the subject, while objective measures require little subject involvement. The most used objective methods include fundus reflectometry and fundus autofluorescence. [53] The most widely used subjective method is heterochromatic flicker photometry (HFP). As HFP is widely used and has low participant invasiveness it will be explored in more detail here.

Traditionally, HFP is the presentation of light stimulus to a subject's fovea at two alternate wavelengths, a short blue wavelength and longer green wavelength. The colours are alternated as

such a frequency and luminance that they appear to be a flickering light. The ratio of blue to green light is adjusted by the subject, clicking a button until the observed flickering is resolved. This occurs at the point of equal luminescence between the blue and green lights. The change to blue light (BL) intensity required is correlated to the degree of macular pigment, as the macular pigment absorbs a portion of the BL. This procedure is then repeated at the peripheral macula, where MP is low, and compared to the central measure to achieve a log ratio that is MPOD. A newer variation of this method uses a series of pre-set green-blue light ratios, at a constant luminescence, presented above the critical fusion frequency. The flicker rate is gradual reduced from 60 hertz in 6 hertz increments until the subject indicates by pressing a button the appearance of the target flickering. This is repeated for all pre-set ratios centrally (1° area of the macula centre) and peripherally (8° eccentricity from the centre of the macula), to compare and estimate the minimum points obtained for an MPOD value. [51] HFP has been shown to have good test-retest reliability and was validated though comparison of MPOD measures to profiles of donated human retinas. [53, 203]

As HFP is a psychophysical measure it is important to ensure participant error is minimised. A study conducted by Howells, Eperjesi and Bartlett [47] successfully explored a protocol to improve the repeatability and reliability of this newer HFP method variation. The study protocol utilised the same research investigator and verbal explanation to standardise information delivery to participants. The protocol recommendations from this study indicated that each participant should perform the central and peripheral test twice. A third test should be conducted if there is a ≥0.4 decibel difference between the minima of a curve of the two readings for either the central or peripheral measure. Alternatively, a third measure should be conducted if the shape of the curve generated by the central or peripheral measurement is not the optimal 'V' shape and is manually adjustable. A curve that is has multiple points close together as the minimum, a 'U' shape, may be able to be adjusted by manually selecting what the investigator determines to be the minimum point. Lastly, in the case that a curve has a poorly defined minimum the measure should be discarded for calculation of the participants mean MPOD. [47] Understanding where errors can occur and minimising error in the measure of MPOD ensures any attempt to explore the relationship with ED use and dietary L/Z intake is optimised.

3.1.2 Background for developing a specific tool to monitor electronic device use

To develop an ED use specific questionnaire, behavioural research methodology can be a source of inspiration to draw upon. The use of EDs is a daily behaviour with patterns of use likely to have similarities with dietary and sedentary behaviours. Factors to consider may include bias of the tool selected, timeframe of participant recall, question structure, and methods for new tool validation.

3.1.2.1 Objective and subjective methods

In the context of monitoring ED use for impacts on MPOD, the ideal method to monitor ED use behaviours would be objective. An objective measure specific to ED use, such as a biological marker, is not currently available. Other subjective methods available are observation and questionnaires. As previously discussed, (section 2.1.1) observation is a desirable option for reducing forms of within-person bias. However, observation is time intensive and may not be representative of free-living use. Therefore, observation may have reduced generalisability and capacity to be repeated in future research. Capture of ED use with an adapted FFQ or sedentary behaviour questionnaire could be suited to investigating the impacts of chronic ED use due to the longer recall timeframe. Although repeat use tools such as a diary could also be performed, this may be less feasible to complete in large population studies and places a higher burden on the participant. An unvalidated questionnaire, rather than diary, appears to have been used previously by commercial companies. [200] Reflecting on dietary intake methods previously discussed (section 2.1.1), different tools are subject to different biases. These biases include difficulty and inconvenience of estimating volumes or weights of a food, high inter-day variability in intake (also known as within-person random error), reactivity bias, and social desirability bias. [75-77] In the context of ED use this we propose that equivalent biases are likely to be present.

3.1.2.2 Prior research with capture of electronic device use

Equivalence of biases with ED use is suggested from outcomes of a study that developed a questionnaire to monitor near work activities that did include a measure of ED use. [204] Twenty-three university students aged 18–25 years were asked to recall and report hours of near work activities, such as reading, painting, and ED use. The hours of near activity were compared with hours of near activity measured by the RangeLife glasses. The RangeLife device is a pair of glasses that uses an infrared light to detect how close an object is to the eye, accurate to approximately 1.2 m. The glasses are not able to differentiate between near work activities conducted at the same distance. That is, an object 0.2 to <0.3 m from the glasses could be a handheld device or printed material. The glasses were worn by subjects for two days, one weekday and one weekend day. Compared with the glasses the subjects overreported hours of near work activities by approximately 4 hours, $p = 0.002$. Approximately 10 hours/day reported by the questionnaire and 6 hours/day by the glasses. [204] A similar study was later conducted using the same technology called Clouclip. [205] Twenty-five participants aged 22–45 years wore the Clouclip glasses for seven days (5 weekdays, 2 weekend days) and this was compared to hours of near work reported by a questionnaire. Time performing near activities was not significantly different between the questionnaire and Clouclip glasses. Time performing intermediate viewing distance activities was

overreported in the questionnaire compared with the glasses by 4.5 hours / day. [205] The questions addressing ED use in these studies were similar and are available in the supplementary materials for both studies. [204, 205] There was a question about hours of daily ED use for three different device categories: television, computers, and handheld. The question structure in both surveys did not include a specific timeframe for participants to estimate their daily hours of ED use for. An example of specifying a timeframe of recall is, 'In the last 7 days…'. The options for responses were a scale of 0.5-hour increments for one [205], and checkboxes of 1-hour ranges for the other (for example, 1–2 hours, 3–4 hours). [204] The individual ED use hours recorded by participants was not reported in these studies. While not specifically about ED use, these two studies indicate that there are differences in recalling near and intermediate viewing activities between the chosen objective measure and the questionnaire. The overestimation of near and intermediate activity by the questionnaire was suggested to be related to memory recall bias. [204, 205] Therefore, for a new ED use questionnaire a shorter recall timeframe may be most appropriate, for example 3 months rather than 12 months.

3.1.2.3 Electronic device categories

The separation of EDs into categories may be important to capture devices that contribute significantly to this memory recall bias. Separation into categories may also be appropriate to capture how different EDs are utilised, luminance potential of an ED, and distance differences EDs are viewed from. An example of distances an ED may be viewed from can be observed with smartphones and TVs. Smartphones are often held in a hand less than 30 cm from the eye, while a TV may be closer to 200 cm away. The luminance of smartphone displays has been reported to range between approximately 350-750 cd cm^{-2}, and newer organic LED TVs at 540 cd m^{-2} or more. [193] To capture differences in use between different EDs, three categories of EDs may be appropriate: a category for handheld devices such as smartphones and tablets or any other small screen devices able to be held during use, a category for computers such as included laptops and desktop monitors, and a category for TVs including household size TVs and larger screens such as commercial TVs used in movie theatres and social venues.

3.1.2.4 Inter-person variability in electronic device use

The degree and impact of within-person variability in reporting habitual ED use is unknown. An individual may have consistent daily behaviours for ED use, but large fluctuations in daily ED use is also possible. [202] The use of EDs could be likened to physical activity in that it is an activity that may not be performed each day or may be performed at varying times over a day. The impact of within-person variability may be made more complex with differences in behaviour patterns

between population groups or characteristics, such as age. [200] Another example of potential between-person variability is the use of EDs occupationally. Occupational use of EDs may mean differences in total use on weekdays versus weekend days, often referred to as the day of the week effect. [79] The potential for day of the week variation to occur was identified and measured by Williams et al.[204] with the UH NEAR questionnaire. The hours of near activities reported on a weekday versus a weekend were approximately 3 hours more ($p = 0.02$). [204] Potential differences in ED use between weekdays and weekend days should be captured in a new ED use questionnaire.

Related to inter-individual differences may be social desirability bias. It is unknown what societal pressures exist surrounding ED use. That is, whether higher or lower use of ED is any more socially desirable than the other, and if bias shifts in different contexts. Additionally, it is unknown whether perceptions of what is socially desirable may differ between population groups. We propose that biases existent in dietary and sedentary behaviour research are appropriate to consider in the development of a questionnaire tool to monitor ED use.

3.1.2.5 Electronic device use questionnaire recall timeframe

Another factor to consider in the development of an ED use questionnaire is the appropriateness of the timeframe data is captured for. The capture of both short and longer-term ED use should be considered at present because the impact of either timeframe on MPOD is not understood. Regarding the capture of longer-term behaviour patterns, FFQ or screeners are used in dietary methods, and questionnaires like the Minnesota Leisure-Time Physical Activity questionnaire are used in sedentary behaviour measurement. [206, 207] A benefit of the longer recall timeframe in these types of tools is that the likely impact of episodic behaviours are reduced. A bias for consideration is that these types of tools are associated with memory recall bias and can be cognitively difficult for individuals. [75] The cognitive difficulty may influence responses, and refers to the memory and mathematical ability required for individuals to recall and calculate their usual daily hours of ED use. Therefore, a questionnaire with a moderate recall timeframe such as three or six months may be suitable as it may assist to minimise memory recall bias and with repeat use could capture long-term ED use behaviours. [75, 82] Existing dietary or sedentary behaviour questionnaires could be adapted to reflect ED use questions such as those included in the near activity study by Bhandari et al.[205].

3.1.2.6 Electronic device use questionnaire question structure

An element of question design that may assist in reducing cognitive difficulty of recalling ED use is providing prompts or parameters for reporting use. The UH NEAR questionnaire and sedentary

behaviour questionnaires provide examples of this type of question structure. [204, 206] In the UH NEAR questionnaire, participants were asked to report usual hours per day of near activities through ticking of a checkbox. The checkboxes available to tick were ‘not at all’, ‘less than 1 hour’, ‘1-2 hours’, ‘3-4 hours’, ‘5-6 hours’, and ‘7 or more hours’. [204] Conversely, activity diaries may ask participants to record minutes spent performing sitting, light activity, and moderate to heavy activity in short intervals such as 15 or 30 minutes. [206] For a device such as a smartphone that may be used episodically over a single day, asking participants to report use in hourly ranges such as 1-2 hours may be too large. An interval of 0.5 or 0.25 hours may be more reflective of usage patterns. However, an interval of 0.25 hours may not be ideal as a higher mathematical capacity to average and summate daily use may be required. Therefore, 30 minutes may be an appropriate interval for use in an ED use questionnaire attempting to capture long-term use.

3.1.2.7 Other information to capture in an electronic device use questionnaire

Other daily behaviours to inform on ED use reporting and variability in BL exposure from different devices should be captured in a new questionnaire. Daily behaviours that may inform ED use patterns are regularity and duration of physical activity, daily hours of sleep, occupational status, and duties within an occupational role. Variability in BL exposure from different devices is important to consider in the context of how ED use may impact MPOD. Additionally, as EDs are a relatively recent and a sparsely monitored behaviour, capture of change in device use over a more extended timeframe such as 20 years may be of benefit. If ED use impacts on MPOD occur over a long timeframe, capturing use over a large number of years may be necessary. Finally, the introduction of BL filtering ocular lenses and ED applications reduce the amount of BL the eye is exposed to may also influence the relationship between ED use and MPOD. [195, 196] Capturing these additional factors will provide important auxiliary information in a new ED use questionnaire.

3.1.2.8 Validating an electronic device use questionnaire

The method for validation of a new questionnaire to monitor ED use must be thoughtfully selected. Outlined previously (section 3.1.2.1) was that validation against an objective measure for all devices is not available and observation not feasible for translation to larger scale studies. Therefore, relative validity may be most appropriate to validate a new tool. [80] Use of a different tool capturing the same behaviour to compare for any differences in reporting. As discussed earlier (section 2.1.1), tools such as multiple 24-hour diet recalls and food records are often used as the reference method for validation of a new tool. Repeat use of these short-term tools are used to validate FFQ and screeners in a dietary intake setting due to bias differences between the methods and pre-existing validation against biological markers. [80] Applied to ED use behaviour

comparison of hours of device use reported from a questionnaire with a longer recall timeframe could be compared with multiple ED use diaries.

A diary is prospective and may therefore minimise memory recall bias. The structure of sedentary or physical activity diaries would be well suited for adaptation to recording ED use behaviours. Over 24-hours, activity diaries may ask participants to record minutes spent performing sitting, light activity, and moderate to heavy activity in intervals short intervals such as 15 or 30 minutes. [206] To measure ED use, the recording categories such as sitting could be replaced with categories of EDs for example, handheld devices.
Other potential biases to consider with an ED use diary include days chosen for recording, reactivity bias, and recording fatigue. To address potential impacts such as day of the week, diaries would need to be completed on both weekdays and weekend days. [79] Reactivity bias could look to be minimised by using a strategy established in dietary intake measurement, reducing notice participants have that they will be completing the diary. [81] Recording fatigue could look to be reduced by completing diaries on non-consecutive days. [81]

A questionnaire to monitor habitual ED use behaviours is needed to investigate potential impacts of BL exposure from EDs on MPOD. Investigating the impact of ED use on MPOD may subsequently assist in understanding the relationship between dietary L/Z intake and MPOD. A clear relationship between dietary L/Z intake and MPOD will inform potential target dietary intake values. As no objective measures are available, relative validity of a new ED use questionnaire compared with an activity diary adapted for ED use monitoring is a pragmatic option.

3.2 Publication details

Section 3.3 to 3.7 of Chapter 3 includes the manuscript published in BMC Public Health (Journal Impact Factor: 4.5; Quartile 1). Numbering of tables, figures, and references are presented as part of the whole thesis and as such numbering is different to that of the submitted work. No other text in section 3.3 to 3.7 is different to the submitted manuscript.

N. K. Fitzpatrick, V. Chachay, S. Capra, D. Briskey, S. Jackman, A. Shore, Bowtell J. Assessing electronic device use behaviours in healthy adults: development and evaluation of a novel tool. BMC Public Health. 2024;24(1):186. doi: 10.1186/s12889-024-17637-4

3.3 Introduction

Prolonged and chronic exposure to electronic devices, referred to as 'devices' hereinafter, has been identified as an emerging public health issue with implications for conditions such as sleep issues, digital eye strain (also known as computer vision syndrome), myopia, and retinal damage in the eye. [197, 201, 204, 208] The exposure to blue light from device screens has been hypothesized to cause photochemical damage at the macula in the eye. [10, 172] Chronic exposure to blue light from devices has not yet been confirmed as a radiation issue; however, investigation is warranted due to the plausible mechanism for retinal damage supported by animal studies. Photochemical damage to the retina from blue light has been demonstrated in both in vitro and animal experimental studies. [179, 183, 184] Additionally, the light emitting diode form of blue light exposure seen from devices is a relatively new environmental exposure with no longitudinal data available on the potential impacts.

Devices in this study refer to those with display screens such as smartphones, tablets, computers, and televisions. The impact of long-term human blue light device exposure has not yet been investigated, in part because no validated methods to measure this human exposure exist. Reports to date have been with unvalidated interview or questionnaire methods, and often through commercial entities. The 2019 Deloitte mobile and media report is one such example and indicates that the uptake and use of devices has increased since 2017. The report indicated that nine in 10 Australians own a smartphone, and average daily use is three hours. [198, 199] The Deloitte Media and Entertainment Consumer Insights 2023 report indicated that Australian adults spend 3 hours and 54 minutes per day watching videos, 54 minutes per day browsing social media, and 30 minutes per day playing video games. [209] Another commercial report, the United Kingdom (UK) based Ofcom 2018 Communications Market Report, indicated from self-reported recall that one in five adults spent a weekly average time online (activities involving internet use) of more than 40 hours. [200]

The use of devices appears to be widespread; however, behaviours surrounding the types of devices being used and habitual patterns of use are unclear. A specific and valid method for monitoring device use behaviours is needed to understand behaviour patterns. A method is also needed to determine the clinical implications of the potential negative impacts of blue light exposure, such as myopia and macular degeneration risk. [197, 201, 204] In addition to ocular health implications, a method to monitor device use behaviours may have application in other areas of research such as use of devices as assistive technology, social equity, and psychosocial impacts on interpersonal relationships. [210-212] This study describes the development and validity evaluation of a novel tool to monitor usual device use titled the Electronic Device Use Questionnaire (EDUQ). The study

aims were to develop the EDUQ and validate daily hours of device use reported by the EDUQ against multiple 24-hour electronic device use diaries (24DUD) in healthy Australian and UK adults.

3.4 Methods

3.4.1 Recruitment

A convenience sample of adults residing in Australia and the UK was recruited via electronic and paper advertisements. Australian participants were recruited between August 2020 and June 2021, and UK participants were recruited between August 2021 and November 2021. Eligible participants were healthy adults 18 years or older able to complete online questionnaires. The exclusion criteria were no English language literacy and visual, hearing, or physical impairment that prevented online questionnaire completion. This study was approved by the University of Queensland Low and Negligible Risk ethics committee and the Sport and Health Sciences ethics committee at the University of Exeter (#2020001764). All participants provided written informed consent.

3.4.2 Electronic device use questionnaire development

As no literature specifically addresses valid capture of screen time from devices, the literature on research in physical activity, dietary intake, and myopia was drawn upon. [75, 204, 206] Five key factors for consideration in the development of the questionnaire emerged from this literature: the categories of devices, day-to-day variability in device use, timeframe of participant recall, question structures to report device use, and other daily behaviours that may inform device use. [75, 204, 206]

The categories of devices aimed to capture differences between devices in patterns of use, device screen luminance, and distance of viewing from device. [193] The luminance of a device and viewing distance from a device during use may play a role in their impact on ocular health, for example smartphones may have a lower luminance compared to a television but are held a shorter distance from the eye. [193] Thus, three logical categories were handheld devices (for example, smartphones and tablets), computers (for example, laptops and desktop monitors), and televisions (including household and commercial sizes). This grouping was adopted from the device groupings in the three device use related questions in the University of Houston Near work Environment Activity, and Refraction (UH NEAR) questionnaire. The UH NEAR was developed to investigate near viewing activities such as reading, writing, and use of devices. [204]

Day-to-day variability in device use is a likely bias equivalent to that established in other areas of behaviour research, such as dietary intake. [79, 200, 202] As with dietary intake, the day-to-day variability may be impacted by participant characteristics such as age and occupational status. [200]

The need to capture day-to-day variability is also supported by prior research, where it has previously been estimated using the UH NEAR questionnaire that device use is approximately three hours more on a week day compared to a weekend day. [204]

The timeframe of participant recall was selected with consideration for the unknown degree of variability in device use behaviours, potential for episodic device use, and memory recall bias. The established biases and recall timeframe used in dietary intake and sedentary behaviour research informed the timeframe of recall for the EDUQ. [206, 207] A moderate length recall timeframe of 3 months was selected to balance the attempt to capture habitual device use whilst reducing the impacts of episodic behaviours, mathematical cognitive and calculation difficulty, and memory recall bias. [75, 76, 82]

Question structure was considered so that use of devices over a day was captured. [204, 206] A parameter of 30-minute intervals for reporting daily hours of use for each device was selected. A pre-determined range was selected to assist reducing the cognitive difficulty of recalling the behaviour. [204, 206] The UH NEAR questionnaire utilised 60-minute intervals and the questionnaire returned high rates of overreporting compared to glasses that recorded distance of the eye from an object over the same recall period. [3] Activity diaries utilised 15- or 30-minute intervals. [206] While fifteen-minute intervals may be appropriate for reporting episodical use of devices such as smartphones, however 15-minute intervals may also require higher mathematical computational and averaging capacity, which may negatively impact the accuracy of recall. [206] Thus, a 30-minute interval was selected for reporting hours of device use.

The final factor considered was other daily behaviours that may inform device use. As a novel area of behaviour research, other daily behaviours, and participant characteristics may be important to understand device use patterns. Auxiliary daily items included were physical activity, sleep, occupational status, duties within occupational role, history of device use, use of blue-light filtering ocular lenses and device settings, and device-generated reports of daily use. [195, 196][204]

An internal test of face validity was conducted with two members of the research team (S.C. and V.C.) and a convenience sample of 21 Australian and UK individuals known to N.F. who volunteered to read, fill out and discuss the EDUQ. [213] Discussions with respondents indicated all individuals understood what an electronic device is and that daily hours of device use were requested for a weekday and weekend day separately. All but two individuals reported the 30-minute increment for reporting device use to be appropriate, while two respondents suggested a 15-minute increment could improve the EDUQ. Three changes were made to the EDUQ following respondent feedback. One change was clarifying what constitutes physical activity through providing examples of activities. Another addition was including the daily hours of use as reported by the devices' own data capture system (e.g. on a smartphone). The last change was providing

examples of lutein and zeaxanthin containing supplements to assist recall of supplement intake. The final EDUQ contained four sections with a total of 22 questions (Appendix C-1). Section one contained nine questions relating to personal characteristics and medical history, including age, gender, country of residence, and ocular health. Section two contained three questions relating to education and occupational status. Section three contained five items relating to device use. Three categories of devices with screens were included: handheld devices (for example, smartphones and tablets), computers (for example, laptops and desktop monitors), and televisions (including household and commercial sizes). The items included reporting usual daily hours of device use on a weekday and a weekend day, change in daily device use over the last one to 20 years, and use of visual correction glasses with or without a blue light filter. Section four contained four questions relating to the use of sunglasses, physical activity and sleep on weekdays and weekend days.

3.4.3 Twenty-four hour electronic device use diary development

The 24DUD was developed to perform relative validity testing with the EDUQ, as no other tools designed specifically to monitor device use existed. The diary was developed by adaptation of a prospective physical activity diary used by Cartmel et al.[206]. This diary was modified to reflect electronic device use. Titled the '24-hour electronic device use diary', the diary recall timeframe was prospective from 00:00 to 23:59 and contained 15-minute intervals in which participants recorded use of handheld, computer, and television devices (Appendix C-2).

3.4.4 Data collection

Over eight weeks, recruited participants completed eight (one per week) diaries and three EDUQs (Figure 3-1). The day for diary completion was randomly allocated at baseline within the constraints that two of the eight diaries were scheduled for weekend days and the remainder for weekdays. The EDUQ was completed at baseline and at the conclusion of weeks four and eight. Participants were notified by email when a diary or EDUQ was to be completed. The EDUQ and diary were hosted on Checkbox Survey® for Australian participants and Qualtrics XM® survey platform for UK participants.

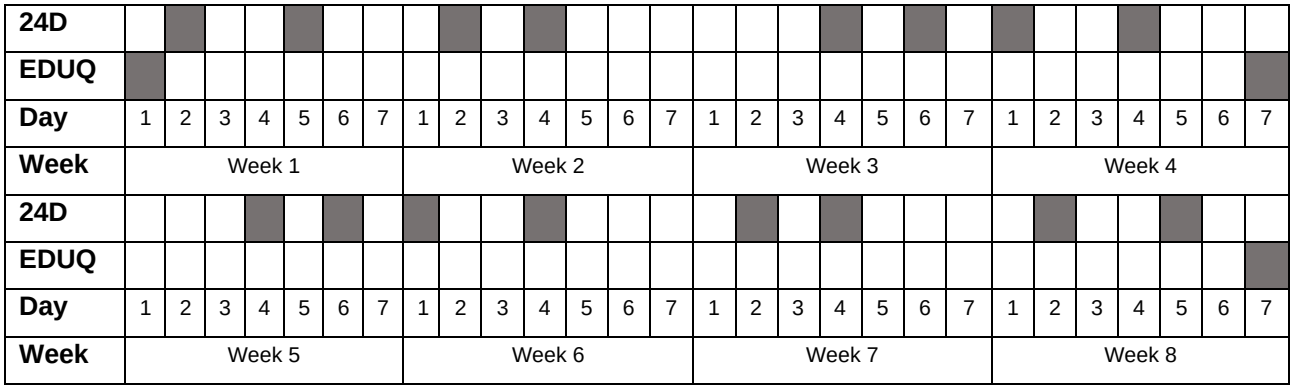

Figure 3-1 Questionnaire and diary schedule of data collection.
The day of the week for the measurement of 24-h electronic device use diaries varied randomly between participants. Abbreviations: 24D, 24-h electronic device use diary; EDUQ, Electronic Device Use Questionnaire

3.4.5 Data processing

In the EDUQ, mean daily hours of device use for each device category cumulatively and separately was derived using:

EDUQ mean daily hours = ((Weekday device use × 5) + (Weekend day device use × 2)) ÷ 7 (Appendix C-3). In the diaries, the mean daily hours of device use for each device category cumulatively and separately were derived using

Diary mean daily hours = Sum hours from all completed diaries ÷ number of diaries completed.

3.4.6 Sample size

In the absence of a validated tool or literature on device use, physical activity and near viewing activity questionnaire literature was referenced to determine a sample size. One study demonstrated that 24 adults aged 66-88 years was a sample size able to indicate reporting trends between two tools with the comparison of a physical activity questionnaire to an activity diary. [206] The validation study of the UH NEAR questionnaire by Williams et al.[204] had a sample size of 23 participants and was able to obtain an indication of questionnaire validity but suggested that a larger sample size would be beneficial for future studies. Thus, a minimum goal sample size of 40 participants per country (Australia and UK) was determined.

3.4.7 Statistical analyses

Statistical analysis was conducted using SPSS (28.0.0.0). [163] Participant responses to each EDUQ were screened for likely overreporting by summing the responses to daily hours of device use, physical activity, and sleep. A sum over 168 hours/week was flagged and investigated further, as participants could have overreported one or all three behaviours. Other participant

characteristics, such as occupation, were reviewed to determine the feasibility of high device use contributing to the more than 168 hours/week. Participants with 172 or less hours/week and plausible characteristics to explain high device use were included in the questionnaire analysis. Any participant with EDUQ reporting over 168 hours per week and no feasible explanation was excluded. The 24DUDs were assumed to be accurate and included as long as the participant reported one or more EDUQ that passed the screening process for overreporting.

Data normality was tested with the Shapiro–Wilk test. Differences between cohort participant characteristics and device use were tested with a Chi-squared test, two-tailed independent samples t–test or Mann–Whitney U–test. In both cohorts, a Bland–Altman plot analysis of the mean daily hours of device use (all categories combined) was performed to compare the third EDUQ and six or more combined 24DUDs. [164, 165] The third EDUQ was used so that the timeframe of recall for EDUQ device use aligned with reporting from the diaries. The same Bland–Altman plot analysis was also performed for each device category individually. Participants with fewer than six 24DUDs were removed from the questionnaire analysis. Six rather than eight 24DUDs were chosen to increase the data available for analysis, as only seven UK participants had completed all eight diaries. Six diaries were determined to be appropriate, as no significant difference was found between the complete or partially complete larger Australian dataset for the parameters required for the Bland–Altman plot analysis. If the difference between tools was not normally distributed, the data were log base 10 transformed to achieve normality for Bland–Altman plot analysis.

Cronbach's alpha and two-way mixed effects model absolute intraclass correlation coefficient was performed for test-retest reliability between the first, second, and third EDUQ. Normally distributed data are presented as the mean ± standard deviation, and nonnormally distributed data are presented as the median and 25^{th} to 75^{th} percentile. The results were considered statistically significant at $p<0.05$.

3.5 Results

Fifty-six Australian and 24 UK participants enrolled in the study. Across the third EDUQ and diaries, six Australian and 11 UK participants had implausible EDUQ data or did not complete the questionnaires needed for the validity and reliability analysis (Figure 3-2).

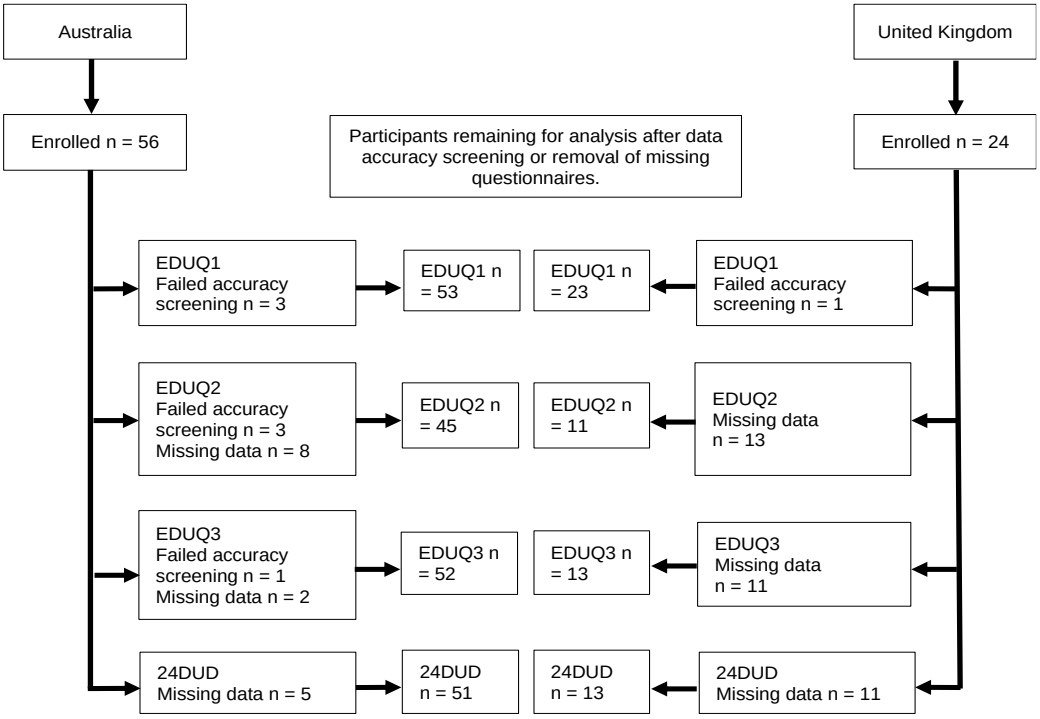

Figure 3-2 Participant flow chart of device use study completion.
Abbreviations: n, number of participants; EDUQ, Electronic Device Use Questionnaire; 24DUD, 24-hour device use diary.

Table 3-1 Australian and United Kingdom participant characteristics

	Median (25^{th} – 75^{th} percentile)		Difference between cohorts [a]
	Australian (n = 56)	UK (n = 24)	
Age (years)	27 (25 – 32)	27 (25 – 52)	p = 0.002
Sex (% female)	68 %	63 %	p = 0.29
BMI (kg/m^2)	24 (22 – 26)	26 (24 – 31)	p = 0.02
Physical activity per week (hours)	5 (3 – 8)	3 (0.5 – 7)	p = 0.06
Sleep per night (hours) (mean ± SD)	7.7 ± 0.73	7.0 ± 0.97	p = 0.002
Education (% completed higher education)	88%	54%	p<0.001
Occupational status (% student, % employed)	49%, 46%	25%, 58%	p = 0.07

Difference between cohorts tested by Mann-Whitney U-test for continuous variables and Chi-squared test for categorical variables. Abbreviations: n, number of participants; UK, United Kingdom; BMI, body mass index; SD, standard deviation.

The median age of the Australian participants was 27 (25 – 32) years, 68% were female, and 88% had a tertiary education (Table 3-1). The median age of the UK participants was 27 (25 – 52) years, 63% were female, and 54% had a tertiary education. Significant differences in age (p = 0.002),

body mass index (p = 0.02), and education status (p<0.001) were present between the Australian and UK cohorts.

The mean Australian device use reported from the EDUQ ranged from 8.9 to 9.6 hours/day. The mean UK use ranged from 11.1 to 11.7 hours/day (Table 3-2). Computers were the device category with the highest mean daily use across both cohorts and tools. Australian reported hours of use for all device categories individually and combined were significantly correlated between the third EDUQ and 24DUDs (Table 3-3). Of both cohorts, the strongest correlation was in the UK cohort with handheld device use, r = 0.93, R^2 = 0.87 (p<0.001).

Table 3-2 Daily hours of electronic device use reported from the Electronic Device Use Questionnaire and mean of combined 24-hour device use diaries in the Australian and United Kingdom cohorts

Tool	Device category	Australia		United Kingdom		Cohort comparison [a]
		n =	Daily Use (hours)	n =	Daily Use (hours)	
EDUQ 1	All devices	53	8.9 ± 3.16	23	11.4 ± 3.25 [b]	p = 0.002
	Television		1.1 (0.50 – 2.75)		2.4 (1.50 – 4.00) [c]	p = 0.008
	Computer		5.1 (3.40 – 6.60) [d]		4.6 ± 2.98	
	Handheld		2.3 (1.29 – 3.18)		3.2 (2.00 – 6.64)	p = 0.048
EDUQ 2	All devices	45	9.2 ± 3.08 [e]	11	11.7 ± 2.60 [f]	p = 0.01
	Television		1.5 (0.61 – 2.57)		2.0 ± 1.51	
	Computer		4.7 ± 2.17		5.8 ± 2.64	
	Handheld		2.8 ± 1.65		3.8 ± 3.05	
EDUQ 3	All devices	53	9.6 ± 2.61 [g]	13	11.1 ± 2.22	p= 0.04
	TV		1.5 (0.50 – 2.57)		2.5 ± 2.11	
	Computer		4.9 ± 1.76 [h]		4.8 ± 3.42	
	Handheld		3.0 (1.68 – 3.79)		3.9 ± 3.12	
Mean 24DUD	All devices	51	7.9 ± 1.75 [e, g]	13	9.3 ± 2.21 [b, f]	
	TV		1.5 (0.90 – 2.38)		1.6 ± 1.55 [c]	
	Computer		4.0 ± 1.78 [d, h]		4.0 ± 3.46	
	Handheld		2.3 ± 1.31		3.6 ± 3.4	

Data presented as mean ± SD or median (25th – 75th percentile). Differences between countries tested by two-tailed independent samples t-test or Mann–Whitney U-test. Within country differences between questionnaires for a device category tested by two-tailed independent samples t-test or Mann–Whitney U-test and indicated by matching superscript letter (for example, [b]). Abbreviations: EDUQ Electronic Device Use Questionnaire, 24DUD 24-h electronic device use diary, n Number of participants. [a] Blank cell indicates non-significant differences between cohorts for row variable. [b] p = 0.049. [c] p = 0.047. [d] p = 0.02. [e] p = 0.02. [f] p = 0.04. [g] p < 0.001. h p = 0.007.

For both cohorts, the Bland–Altman plot analysis indicated poor agreement of daily hours of ED use between the third EDUQ and combined 24DUDs with modest mean differences but large 95% limits of agreement (Table 3-3). The Australian cohort indicated slightly better agreement than the UK cohort, with a mean difference of 1.54 hours and 95% limits of agreement from -2.72 hours to 5.80 hours. There were no trends in the direction of differences between tools (Figure 3-3).

Table 3-3 Bland-Altman plot analysis outcomes of daily hours of electronic device use reported from the Electronic Device Use Questionnaire and 24-hour device use diaries

			Bland–Altman Plot Analysis (hours/day)			Correlation between reported use
		Device category	Mean difference (95% CI)	Lower 95% LOA (95% CI)	Higher 95% LOA (95% CI)	
Australia	EDUQ3 vs 24DUD (n = 50)	All devices	1.54 (0.00 – 3.08)	-2.72 (-4.26 – -1.18)	5.80 (4.26 – 7.34)	r = 0.54, R^2 = 0.29 p<0.001
		Television [a]	0.08 (0.00 – 0.16)	-1.59 (-1.67 – -1.51)	1.74 (1.67 – 1.82)	r = 0.79, R^2 = 0.64, p<0.001 [b]
		Computer	0.95 (0.00 – 1.90)	-2.28 (-3.23 – -1.33)	4.18 (3.23 – 5.13)	r = 0.57, R^2 = 0.33, p<0.001
		Handheld [c]	0.14 (0.00 – 0.30)	-0.32 (-0.40 – -0.22)	0.91 (0.67 – 1.18)	r = 0.80, R^2 = 0.64, p<0.001 [b]
UK	EDUQ3 vs 24DUD (n = 12)	All devices	1.98 (0.00 – 3.97)	-2.80 (-4.78 – -0.87)	6.77 (4.78 – 8.75)	r = 0.44, R^2 = 0.19, p = 0.16
		Television	0.72 (0.00 – 1.45)	-1.54 (-2.26 – -0.82)	3.76 (3.03 – 4.48)	r = 0.57, R^2 = 0.33, p = 0.05
		Computer [c]	0.15 (0.00 – 0.32)	-0.77 (-0.80 – -0.73)	4.67 (3.93 – 5.52)	r = 0.84, R^2 = 0.71 p = 0.001
		Handheld [c]	0.26 (0.00 – 0.60)	-0.36 (-0.49 – -0.19)	1.49 (0.97 – 2.14)	r = 0.93, R^2 = 0.87, p<0.001

Australian cohort EDUQ3 and 24DUD all devices: SEM = 0.31, t value (49 df) = 5.01. Australian cohort EDUQ3 and 24DUD TV: SEM = 0.12, t value (49 df) = 0.65. Australian cohort EDUQ3 and 24DUD Computer: SEM = 0.23, t value (49 df) = 4.08. Australian cohort EDUQ3 and 24DUD Handheld: SEM = 0.02, t value (49 df) = 3.57. UK cohort EDUQ3 and 24DUD all devices, SEM = 0.70, t value (11 df) = 2.81. UK cohort EDUQ3 and 24DUD TV, SEM = 0.73, t value (11 df) = 2.17. UK cohort EDUQ3 and 24DUD Computer, SEM = 0.10, t value (11 df) = 0.60. UK cohort EDUQ3 and 24DUD Handheld, SEM = 0.04, t value (11 df) = 2.34

Abbreviations: CI Confidence interval, EDUQ Electronic Device Use Questionnaire, n Number of participants, 24DUD 24-h electronic device use diary; LOA, limit of agreement; SEM, standard error of the mean; df, degrees freedom; UK, United Kingdom [a] Indicates the analysis was performed with a difference that was not normally distributed and data transformation did not improve. [b] Indicates Spearman's rank correlation test rather than Pearson. [c] Log base 10 transformation of data required for difference between tools to be normally distributed, values reported are back transformed

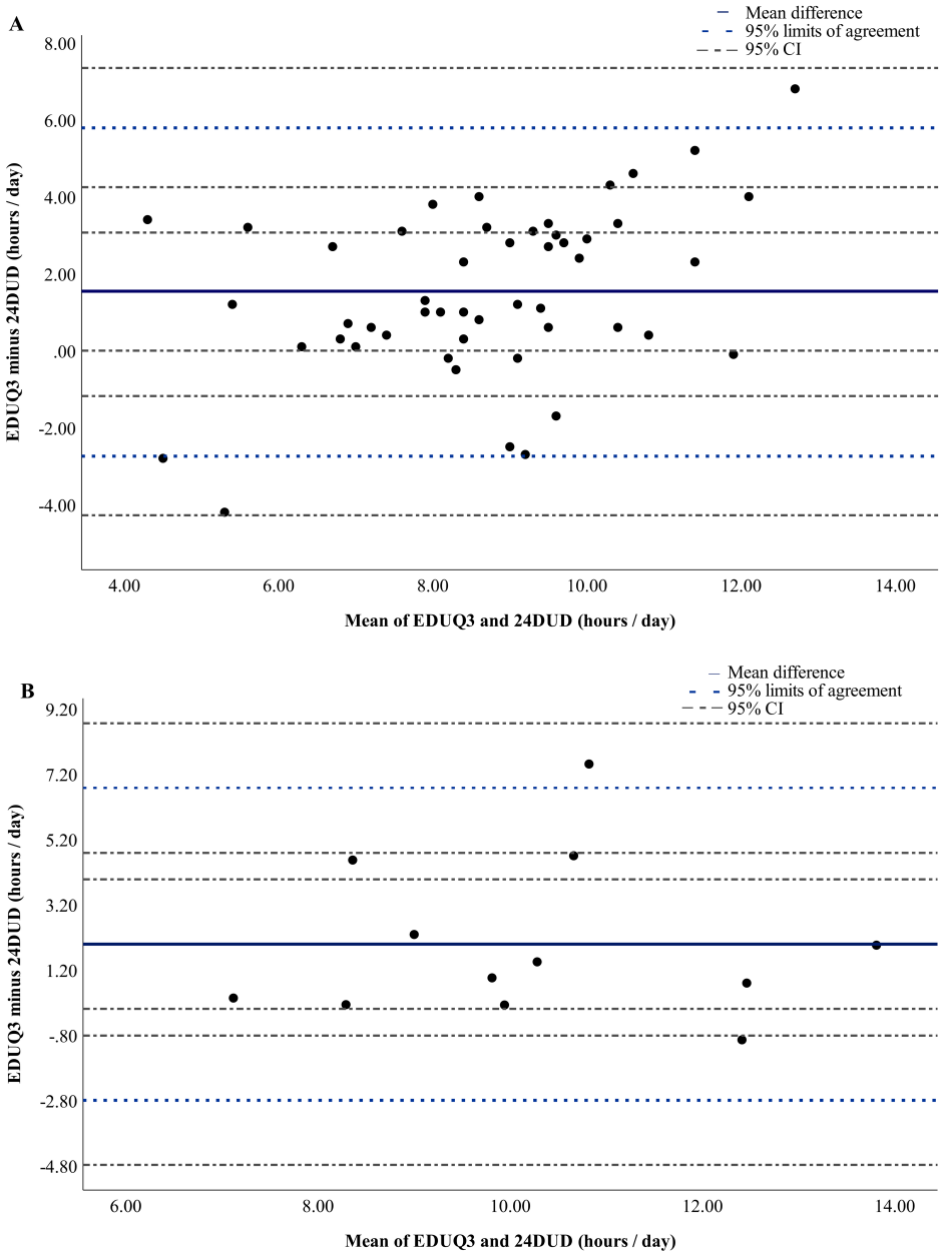

Figure 3-3 Bland–Altman plot analysis, EDUQ3 all devices combined compared with 6 or more 24-hour diaries.
A, Australian cohort. B, United Kingdom cohort. Abbreviations: EDUQ3, third Electronic Device Use Questionnaire; 24DUD, 24-hour device use diaries.

The three EDUQs in the Australian and UK cohorts indicated moderate to high test-retest reliability. In the Australian cohort, the highest test-retest reliability was between the second and third EDUQ, with a Cronbach's $\alpha = 0.91$ and a two-way mixed effects model absolute intraclass correlation coefficient of 0.91. In the UK cohort, the equal highest test-retest reliability was between the first and third EDUQ and the second and third EDUQ, both with a Cronbach's $\alpha = 0.92$ and a two-way mixed effects model absolute intraclass correlation coefficient of 0.92 (Table 3-4). Despite these results, the EDUQ had a poor ability to rank participants into tertiles by daily hours of device use. In the Australian cohort, there was 25% to 36% misclassification of participants into

adjacent or opposite tertiles when comparing the first, second, and third EDUQs. Additionally, when ranked by tertiles determined by the diaries, there was 50% misclassification of participants with their third EDUQ response.

Table 3-4 Test-retest reliability of the three Electronic Device Use Questionnaires completed with all device categories combined

		n =	Cronbach's α	Absolute ICC (95% CI)	p value
Australia	EDUQ1 vs EDUQ2	44	0.78	0.78 (0.60 – 0.88)	<0.001
	EDUQ1 vs EDUQ3	50	0.78	0.78 (0.61 – 0.87)	<0.001
	EDUQ2 vs EDUQ3	44	0.91	0.91 (0.84 – 0.95)	<0.001
UK	EDUQ1 vs EDUQ2	11	0.79	0.80 (0.25 – 0.95)	0.01
	EDUQ1 vs EDUQ3	13	0.92	0.92 (0.74 – 0.98)	<0.001
	EDUQ2 vs EDUQ3	11	0.92	0.92 (0.71 – 0.98)	<0.001

Abbreviations: UK, United Kingdom; EDUQ, Electronic Device Use Questionnaire; n =, number of participants; ICC, intraclass correlation coefficient; CI, confidence interval

3.6 Discussion

The novel EDUQ was developed and evaluated against multiple 24DUDs in adults located in Australia and the UK. Predetermined limits of agreement did not exist on which to benchmark the validity of the EDUQ. Validity was therefore determined by whether the EDUQ agreement with the diaries was such that the EDUQ would be able to capture differences in device use in an intervention or observational study. The poor agreement observed between the third EDUQ and diaries indicated that the EDUQ is not yet valid for use (Table 3-3). In the Australian cohort, the mean difference (95% limits of agreement) was 1.54 hours/day (-2.72 hours/day to 5.80 hours/day). The range between the limits of agreement was 8.5 hours, which is nearly equivalent to the mean daily device use of 7.9 – 9.6 hours/day measured from the two tools in this cohort (Table 3-2). The moderate to high test-retest reliability suggests that the EDUQ is reliable. However, the EDUQ had a poor ability to rank participants by daily hours of device use into tertiles between the first, second, and third EDUQ, confirming its inadequate validity. The differences in reported combined device use between the third EDUQ and diaries appear to be related to an accumulation of participant misestimation within each device category. Additionally, there appears to be no clear trends or predictability in the direction of reported differences across the spectrum of daily device use.

This is the first study to the author group's knowledge that has developed and reported total daily hours of device use. As such, there is no existing peer-reviewed research available on total daily hours of device use to compare against. This study can be compared with prior commercial reports

that use unvalidated interview and questionnaire methods. The 2019 Deloitte mobile and media reports indicated that the average smartphone use for Australians is 3 hours/day, and the average television use is just over 3 hours/day. [198, 199] In this study, the daily hours of handheld use were similar or lower, and television use was lower than that of the commercial report. The median daily handheld device use reported by the third EDUQ was the same at 3.0 hours, and the mean from the 24DUDs was 0.7 hours less. The median daily television use reported by the third EDUQ and 24DUDs were both approximately 1.5 hours less. The UK-based Ofcom 2018 Communications Market Report indicated that one in five adults spend more than 40 hours/week online on the internet, including all devices. [200] In the first EDUQ, the UK cohort indicated a mean of 79.8 hours of device use per week, approximately 40 hours more per week. However, this is inclusive of both online and offline activity. Therefore, the discrepancy may in part be explained by differences in the hours of online and offline device use. The discrepancy and outcomes of this study indicate that offline use may constitute a significant portion of daily device use. The discrepancy may also be explained by usual daily hours of device use continuing to increase. Compared to 1 year ago 32% of Australian and 50% of UK participants indicated an increase in ED use. In contrast, compared to 5 years ago, 72% of Australian and 88% of UK participants indicated an increased in their ED use (Appendix C-4). Reasons for change in device use reported by participants included change to work or study requirements, increased accessibility to devices, increased functionality of devices (e.g. online newspapers), and more engagement with social media.

One reason for the poor agreement between the EDUQ and diaries may be the difference in intervals provided for participants to report their device use between the EDUQ and diaries. Participants could report hours of device use in 30-minute intervals in the EDUQ and 15-minute intervals in the diaries. The larger intervals in the EDUQ may have contributed to the higher mean daily hours of device use reported by the EDUQ compared to the diaries. Future studies should consider closer alignment reporting intervals between tools, for example, reporting intervals of 15 minutes for both the EDUQ and diary.

Another reason for the poor agreement is likely the memory recall bias of recalling device use retrospectively with the EDUQ. Memory recall bias is well established in other areas of behaviour research, such as dietary intake. [75, 82] The presence of memory recall bias with recalling device use is also supported by prior research investigating daily hours of ‘near and intermediate activity’ with the UH NEAR questionnaire. [204] Near and intermediate activity refers to the distance an object is from the eyes and may include paper reading, device use, painting, writing, or playing board games. The mean of the questionnaire-captured recall of near and intermediate activities was reported to be 10.34 ± 0.85 hours/day but only 6.25 ± 0.39 hours/day when captured from objective

infrared glasses. [204] While there are limitations to the sensitivity of the objective measure, such as reduced accuracy at distances over 1 meter, it highlights the likely impact of memory recall bias, in particular, overreporting. The presence of memory recall bias is also supported by the minimal utilisation of devices' own data capture system reports by participants included in the Bland-Altman plot analysis between the EDUQ and 24DUD. Of the combined Australian and UK cohort EDUQ3 data, 68% of participants provided outcomes of device system reported screentime (predominantly smartphone reports), but only three participants indicated using these device reports to inform their answers to questions related to usual daily hours of device use. This suggests participants predominantly relied on memory to estimate daily hours of device use. The utilisation of the device reports did not appear to improve the agreement between the EDUQ and 24DUD, with similar differences occurring for these three participants than for all others. Whilst memory recall bias was hypothesised to be likely associated with the EDUQ during development, the magnitude of impact appeared far greater than anticipated. To evaluate memory recall bias, comparison of the EDUQ against a method such as direct observation may be required.

The poor agreement between the EDUQ and diaries may also indicate that eight 24DUDs are not adequate to capture 'usual' device use. With dietary intake 24-hour recalls, it is known that increasing the number of recalls enables better capture of fluctuations in dietary intake, and thus, outcomes are more likely to be reflective of habitual intake. [68] Daily device use has high potential for day-to-day variability, as demonstrated by participants in this study. For example, one participant with a mean daily use of 6.6 hours from eight 24DUDs reported only 0.5 hours in one 24DUD (handheld device use) and 11.7 hours in another 24DUD (5.58 hours television, 4.00 hours computer, 2.12 hours handheld). It may be that a higher number of 24DUDs are needed to be representative of usual device use. Future studies may consider more days of diary capture or adapting dietary intake methods for device use such as the prospective dietary intake method of a three- or seven-day food record, or a diet history which includes in-depth retrospective capture by interview. In-depth interviewing or continuous capture may help to understand how device use varies between consecutive days. Additionally, future studies could look to investigate opportunities for using reports from the devices' own data capture systems to support monitoring of behaviours across all device types and days of the week. In the present study smartphone and tablet reports were most utilised by participants. With any method selection, participant access to device reports, burden, and reactivity bias with a greater recording period are important considerations. [81] Future research may benefit from providing training or support to participants in how to efficiently record device use. Continued research to improve the validity of the EDUQ, or a similar questionnaire, would be beneficial, as it has the potential to be applied in multiple research areas. As mentioned

earlier, it is of particular interest to understand any impacts of blue light exposure on macular health. [172] The EDUQ could also have applications in other areas of research interested in how device use may relate to population behaviours such as sleep and physical activity or psychological areas such as depression and body dissatisfaction. [214, 215]

Multiple reasons may have contributed to the poor agreement between the EDUQ and 24DUD in this study. As a novel field of research, future studies looking to advance the validity and reliability of measurement of electronic device use behaviours may consider developing new instruments through grounded theory methodology. [216, 217] As seen in the present study, daily device use behaviours appear to be highly variable within and between individuals. Engaging with relevant population groups via focus groups and interviews to understand behaviours around electronic device use will likely be useful to inform the development of methods able to accurately capture electronic device use behaviours.

A number of limitations were present in this study. Convenience sampling resulted in a population that was predominantly young, highly educated, and female rather than representative of the general population. The UK cohort was smaller than the goal sample size, and the questionnaire incompletion rate was high. This was a limitation as it limited the ability to determine EDUQ validity through Bland-Altman plot agreement. [213] Future studies should look to increase the sample size and improve participant questionnaire completion rates, for example by reducing the participant burden with high questionnaire frequency. Another limitation was the use of relative validity with two unvalidated questionnaires as the method. Although access to an objective measure was not available, future studies may benefit from validating the 24DUD through comparison with direct behaviour observation or emerging objective technologies such as previously mentioned infrared glasses, known as the Clouclip and RangeLife glasses. [204, 205] Direct behaviour observation was not available as a comparative method in this study due to study design and data collection being conducted during the COVID-19 pandemic.

3.7 Conclusion

This study reports on a novel tool developed specifically to monitor habitual patterns of electronic device use. The EDUQ demonstrated poor validity with poor agreement and ability to rank participants compared with mean daily hours of device use from multiple 24DUDs. Despite poor agreement, mean daily device use between each EDUQ and the 24DUDs were moderately to strongly correlated. This cohort was unable to consistently report similar device use between the third EDUQ and diaries, with misestimation appearing to occur across all device categories. To

improve the validity of device use capture, future studies may benefit from a larger, more diverse sample size, the same reporting intervals for the tools being compared, and consideration of the time of year for data collection, as well as how an objective or direct observation method could be incorporated into the study design.

3.8 Summary

The EDUQ was developed in order to investigate whether a potential relationship between MPOD and EDs (see Chapter 4). The proposed hypothesis for measurement of ED use was that BL exposure may impact MPOD status, and thus be a confounding factor for understanding the relationship between dietary L/Z intake and MPOD. The findings of Chapter 3 address thesis objective 2, the development and validation of a questionnaire to capture usual ED use behaviours. The objective was achieved as the EDUQ was developed. However, the validation component of the objective was not achieved as the EDUQ was not deemed to be valid based on this validation process. Healthy Australian and UK adults were unable to report comparable hours of ED use through the EDUQ compared with multiple device use diaries. However, trends in ED use behaviours over the last 20 years were successfully captured (Appendix C-4). The findings of Chapter 3 do not directly address any of the 9-criteria in Figure 1-1 (page 31). The findings of Chapter 3 do partially address barrier 2 identified in Figure 1-3 (page 61), and support the investigation conducted in Chapter 4.

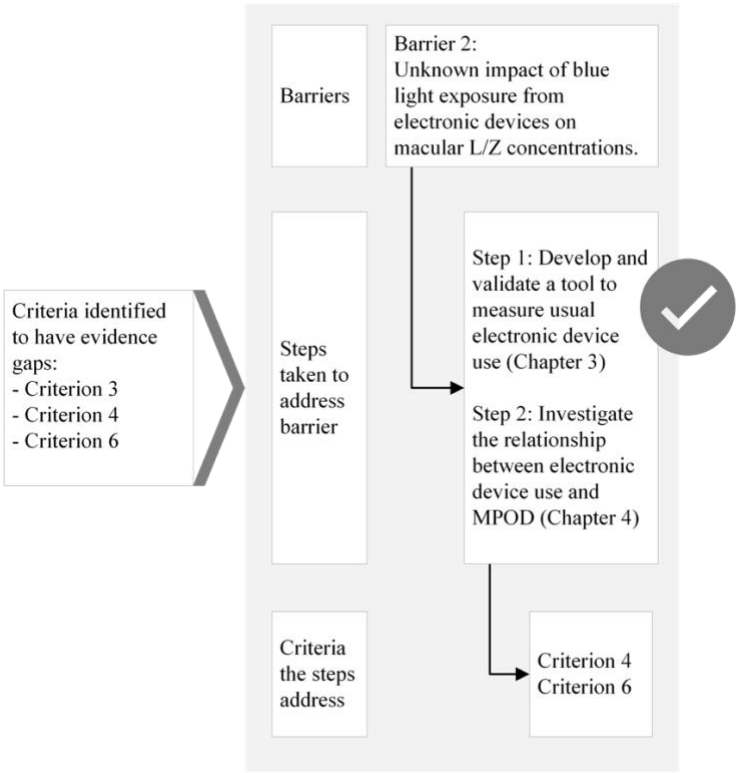

Figure 3-4 Steps addressed as part of Chapter 3 to improve the lutein and zeaxanthin evidence base related to the 9-criteria by Lupton et al. [2]

Chapter 4 Associations between macular pigment optical density, lutein and zeaxanthin dietary intake and plasma concentrations, and daily hours of electronic device use

This chapter describes my original research study addressing thesis objective 3 (section 4.1 – 4.6), to investigate the associations between ED use, dietary L/Z intake, and MPOD in healthy Australian adults, using the newly developed tools. This chapter is written with planned submission to the Asia Pacific Journal of Ophthalmology. It is planned that this chapter will be submitted as a manuscript after the manuscripts submitted as part of Chapter 2 and Chapter 3 are published.

4.1 Introduction

The global incidence of age-related macular degeneration has been reported to be 1.59% and 0.19% for the early and late stages of the condition respectively. [218] In developed countries, age-related macular degeneration is the leading cause of vision loss with global prevalence predicted to increase up to 288 million in 2040. [11, 219] Lutein and zeaxanthin (L/Z) are two dietary carotenoids concentrated in the macula. Increasing supplemental or dietary intake of these macular carotenoids has been associated with decreased risk and slower progression of age-related macular degeneration. [13, 33, 220] The concentration of L/Z at the macula is used as a surrogate marker of macular health, and is known as macular pigment optical density (MPOD). [53]

Exponential uptake of modern technology such as computers and smartphones over the last 30 has resulted in a remarkable increase in artificial blue light exposure amongst all age groups. [198] Animal and in vitro studies have shown that exposure to blue light can cause photochemical damage to the retina. [179, 183, 184] Although a plausible biological mechanism, it is unknown whether chronic exposure to blue light from electronic device sources may influence MPOD status, and increase the risk for retinal damage over time in vivo. [172] A study by Stringham et al.[221] showed that in 18 – 25-year-old healthy adults self-reporting device use more than six hours per day, a significant improvement in MPOD status and visual performance occurs after 6 months of macular carotenoid supplementation. The macular carotenoids are proposed to provide protection to the macula through antioxidant activity and filtering damaging blue light before it reaches other photosensitive molecules. [18, 19, 24, 25, 30] It has not yet been investigated whether a relationship between MPOD status and usual electronic device (ED) use exists. Investigation of this potential relationship is important to determine whether ED use is a risk factor for consideration when observing macular health and risk of age-related macular degeneration.

4.2 Aims

The aim of this study was to investigate the associations between ED use, dietary L/Z intake, plasma L/Z concentrations, and MPOD in healthy Australian adults.

4.3 Methods

4.3.1 Recruitment

In this cross-sectional study design, a convenience sample of participants residing in south-east Queensland, Australia was recruited between September 2020-21 via paper flyers and electronic advertisements. Eligible participants were generally healthy adults 18 to 65 years of age, non-smokers, with no participant reported history of clinically significant medical conditions. The clinically significant medical conditions included, but were not limited to, cardiovascular, neurological, psychiatric, renal, immunological, endocrine (included uncontrolled diabetes or thyroid disease) or haematological abnormalities that were uncontrolled. Participants were excluded if they were a current or past smoker (within last 12 months), or self-reported a diagnosis of epilepsy or serious ocular conditions such as age-related macular degeneration. Procedures for this study were in accordance with the Declaration of Helsinki and was approved by the University of Queensland Human Research Ethics Committee A (#2019002736). Reporting was conducted in accordance with the STROBE statement. [222]

4.3.2 Study protocol

Participants completed a 90-minute scheduled visit at the research facility and online questionnaires within a day post-visit. Participants were asked for a measure of height (stadiometer), weight and body fat percentage by bioelectrical impedance (Tanata BC-541 9-in-1 Body Composition Monitor), MPOD, peripheral venous blood draw, 24-hour diet recall by interview, a dietary L/Z screener questionnaire, and the Electronic Device Use Questionnaire (EDUQ).

The MPOD was measured by heterochromatic flicker photometry using the validated MPS II (Elektron Eye Technology). [51] Participants were provided with the same verbal and visual instruction of how to complete the validated MPOD test by a single study investigator. The test was conducted as per manufacturer instruction. Each eye was measured at least twice using both the central and absolute (peripheral) measures. If a participant's test was rejected, participants were provided feedback and one reattempt. Results from MPOD measures are reported as mean and standard deviation (SD) of two repeat MPOD measures for one eye. As recommended by Howells [47], repeatability of the measurement was improved with use of three factors to determine whether a third measure was included: a ≥0.4 dB difference in the minimum curve reading between measure

one and two for either the central or peripheral curves, a ≥0.09 optical density units (ODU) difference between the absolute (peripheral) MPOD value between measure one and two, or an unclear minimum point on the curve not manually adjustable or deemed a cautionary measure by the MPS II software. Results from each participant's right eye were used for analysis, unless there were insufficient completed and reliable absolute tests in which case results from the left eye were used. If participants failed to complete two absolute tests for both the right and left eye, the results of just the central right eye measurement were used.

Blood draw by peripheral venepuncture was used to measure plasma L/Z concentrations. Collected plasma aliquots were centrifuged for 10 minutes at 3,000 rotations per minute (805 g force, Hettich Zentrifugen Rotina 380 R) and frozen at -80°C until analysis. Thawed plasma L and Z were extracted and analysed by high performance liquid chromatography and photodiode array detection (HPLC-DAD) (Ultimate 3000, Thermo-Fischer Scientific). Analytical methods described by Aebischer et al.[223] and Taibi and Nicotra [224] were used as reference methods. Required chemicals were analytical grade L standard, Z standard, ethanol, hexane, dichloromethane, methanol, acetonitrile, and triethylamine (sourced from Merck Chemicals, Australia). Extracted blood samples were eluted onto a Develosil 5µm RP-aqueous C30 140A, 250 x 4.6mm column with isocratic mobile phase containing methanol (49.96%), acetonitrile (49.96%), and 0.08% triethylamine at a flow rate of 1.2 mL/minute with a 40-minute run time. Detection of L and Z was performed at 445 nm [225, 226]. Identification of L and Z were conducted by comparison with the retention time and absorption spectra of the corresponding analytical standards, spectrophotometric absorbance of the analytical standards was performed, and peaks were established by HPLC-DAD. [227] Standard curves measured for L were linear between the range of 0.1 µg/mL to 100 µg/mL with R^2 values of >0.999. Standard curves measured for Z were linear between the range of 0.1 µg/mL to 10 µg/mL with R^2 values of >0.999. Using the method of standard addition, the assay return was greater than 99%.

The 24-hour diet recall was multiple pass and conducted via interview with the primary investigator (N.F.), an Accredited Practising Dietitian. Participants were asked to recall the amounts of all food and beverages consumed the prior day (midnight to midnight). Food and beverages reported in the 24-hour diet recall were entered into dietary intake analysis software FoodWorks 10 Professional (Xyris Pty Ltd) to calculate energy and nutrient intake, except for L/Z. [228] The USDA food composition tables were used to calculate L/Z intake as Australia did not have comprehensive tables. [138, 229] Participants were screened for accuracy of reporting using their indication of day normality provided and the previously described Goldberg cut off method. [144, 145]

The dietary L/Z screener and EDUQ were emailed to participants and completed online via Checkbox Survey®. The dietary screener has been described in Chapter 2 and was used to capture L/Z intake of 91 foods from the prior month. The dietary L/Z screener also relied on the USDA food composition tables to estimate intake. [138] The structure and data accuracy screening method for the EDUQ has been described in Chapter 3, and was completed to measure usual daily hours of ED use and gather trends of participant device use over the last 20 years, occupational contribution to device use, and weekly sleep and physical activity habits.

4.3.3 Sample size

Sample size calculation was performed based on a MPOD coefficient of variation of 0.187 ODU measured in a sample of 5581 adults using the MPS II (Elektron Eye Technology). [51] A minimum of 84 participants was calculated using a two tailed, random model, linear multiple regression with an alpha error probability: 0.05, power: 0.90, number of predictors: 4. To account for potential 20% participant dropout rate a minimum sample size of 105 participants was determined.

4.3.4 Statistical analyses

Statistical analysis was conducted using SPSS (28.0). [163] Normality testing and descriptive statistics of participant characteristics was performed. Normally distributed continuous variables are presented as mean ± standard deviation (SD) and non-normally distributed data as median and 25^{th} to 75^{th} percentile. Categorical variables are displayed as frequencies and percentages (n, %). No imputation of missing data was performed. Differences between participants with and without missing data were compared by a two-tailed, unpaired t-test or chi-squared test as appropriate. Based on results from normality testing, a two-sided Spearman's rank order correlation or Pearson's product moment correlation tests were conducted between the variables of interest: MPOD, age, sex, usual daily hours of ED use, screener dietary L/Z intake, 24-hour recall dietary L/Z intake, and plasma L/Z concentrations. The associations to MPOD of hours of ED use, dietary L/Z intake, age and sex were assessed using a multiple linear regression analysis. The associations to plasma L/Z of dietary L/Z intake, body fat percentage, age and sex were assessed using a multiple linear regression analysis. Results were considered statistically significant at $p < 0.05$.

4.4 Results

4.4.1 Participant characteristics

Ninety-six eligible Australian adults enrolled with no participants dropping out. Participants were 67.7% female, the age range of participants was 19 – 63 years with a median (25^{th} to 75^{th}

percentile) age of 27 (24 – 39.8) years (Table 4-1). The range of MPOD values was 0.1 – 0.87 ODU, and the mean MPOD was 0.42 ± 0.16 ODU. The left eye was used for nine participants, and the central right eye measurement for three participants. The ODU values of these 12 participants were not significantly different to participants with complete and reliable right eye measurements (unpaired two-tailed, t-test p = 0.77). The MPOD status measured between the left and right eyes were not significantly different (p = 1.0), and were significantly correlated, r = 0.85, R^2 = 0.72, p <0.001. The mean MPOD between men and women was not significantly different and was 0.39 ± 0.14 ODU and 0.43 ± 0.17 ODU respectively. Ten participant plasma L/Z samples were missing, one due to a request not to have a blood sample taken, and nine due a machine failure during analysis. The participant characteristics of these 10 participants were not significantly different to the remaining cohort. Four participant's ED use reports were excluded due to not passing the accuracy of reporting check described elsewhere (Chapter 3, section 3.4). The mean ± SD usual daily hours of ED use for all devices combined was 9.3 ± 3.1 hours/day. Computer was the highest contributing device to ED use. The contribution of food groups to total dietary L/Z intake from the dietary L/Z screener was dominated by vegetables at 91% (Appendix D-1). The top six contributing foods to total L/Z intake were raw baby spinach, cooked broccoli, raw Cos or Romaine lettuce, raw orange carrot, cooked pumpkin, and cooked zucchini (Appendix D-2).

Correlations were observed between the variables MPOD, usual daily hours of ED use (weekday and weekend combined for all devices combined), daily L/Z intake, plasma L/Z, body fat percentage, age and sex. Female participants were more likely to have a higher plasma L/Z concentration, body fat percentage, and be younger than male participants. The only variable MPOD was significantly correlated with was individual and combined plasma L and Z values. The weak correlation with combined plasma L/Z was r = 0.32, R^2 = 0.09, p = 0.002. Usual ED use was not correlated with any other variables. The daily L/Z intake reported from the monthly L/Z screener was significantly correlated with individual and combined plasma L and Z, combined plasma L/Z r = 0.28, R^2 = 0.35, p = 0.008. Plasma L and Z were strongly correlated, r = 0.89, R^2 = 0.90, p = 0.003. The L/Z intake from the 24DR was only weakly correlated with daily L/Z intake from the monthly L/Z screener, r = 0.23, R^2 = 0.05, p = 0.03. After removal of three outlier participants with L/Z intakes of more than 400 mg/month (14.3 mg/day) the association between the monthly screener intake and plasma L/Z remained, r = 0.22, R^2 = 0.06, p = 0.047.

Table 4-1 Participant characteristics

Age (years)	27 (24 – 39.8)
Sex (% female)	67.7
BMI (kg/m^2)	24 (21 – 27)
Body Fat Percentage (%)	27 (8.4)
Hours of Physical Activity / week (hours)	6 (4 – 9)
Hours of sleep / night (hours)	7.6 (7.1 – 8)
Education status (% tertiary educated)	83
MPOD (ODU)	0.42 (0.16)
Combined plasma L/Z concentration (µg/mL)	0.24 (0.15 – 0.31)
Plasma L concentration (µg/mL)	0.15 (0.11 – 0.20)
Plasma Z concentration (µg/mL)	0.05 (0.03 – 0.10)
Dietary L/Z screener L/Z intake (mg/day)	4.6 (2.7 – 7.4)
24-hour diet recall L/Z intake (mg/day)	1.9 (0.9 – 4.9)
EDUQ	
Usual ED use weekday and weekend day combined	
All Devices (hours/day)	9.1 ± 3.1
Television (hours/day)	3.0 (1.0 – 5.0) $^{*, **}$
Computer (hours/day)	8.5 ± 4.1 $^{\dagger, **}$
Handheld (hours/day)	4.8 (3.0 – 7.0) $^{\ddagger, **}$
Usual ED use weekday	
All Devices (hours/day)	10.0 ± 3.4 §
Television (hours/day)	1.0 (0.5 – 2.0) $^{\P, \dagger\dagger}$
Computer (hours/day)	7.0 (4.0 – 8.0) $^{\Vert, \dagger\dagger}$
Handheld (hours/day)	2.0 (1.5 –3.0) ††
EDUQ Usual ED use weekend day	
All Devices (hours/day)	6.5 (5.0 – 8.9) §
Television (hours/day)	2.0 (0.5 – 3.0) $^{\P, \ddagger\ddagger}$
Computer (hours/day)	2.0 (0.5 – 3.0) $^{\Vert, \S\S}$
Handheld (hours/day)	2.5 (1.5 – 4.0) §§
EDUQ 24-hour devices use recall	
All Devices (hours)	8.8 (5.0 – 11.4)
Television (hours)	1.0 (0.0 – 2.5) $^{*, \P\P}$
Computer (hours)	3.3 (0.6 – 7.5) $^{\dagger, \P\P}$
Handheld (hours)	2.3 (1.5 – 3.5) $^{\ddagger, \P\P}$

Unless otherwise specified data presented as median (25^{th} – 75^{th} percentile) or mean ± SD. All characteristics n = 96, plasma concentrations n = 86, EDUQ n = 92. Differences between ED categories and days (weekend, weekday, 24-hour recall) were tested by Independent-Samples Mann-Whitney U-Test. As the EDUQ 24-hour device use recall contains both weekdays and weekend days, no comparison made with usual EDUQ weekend day and weekday totals. Matching symbols of *, †, ‡, §, ¶, $^{\Vert}$ indicates a significant difference between the types of days of ED use (all p values <0.005). Matching symbols **, ††, ‡‡, §§, ¶¶ within a type of day indicates difference between ED categories (excludes all devices combined). Abbreviations: ED, electronic device; EDUQ, Electronic Device Use Questionnaire; L, lutein; MPOD, macular pigment optical density; ODU, optical density units; SD, standard deviation; µg/mL, micrograms per millilitre; Z, zeaxanthin.

4.4.2 Regression model to predict macular pigment optical density

The multiple linear regression to predict MPOD from ED use, screener dietary L/Z intake, sex, and age was not statistically significant, $F(4, 87) = 1.396$, $p = 0.24$, adjusted $R^2 = 0.06$ (Table 4-2, (a)). Statistically, none of the four variables added significantly to the prediction. One assumption was violated with one leverage value greater than 0.2, this participant reported an unusually high L/Z intake of 22.4 mg/day (600 mg/month). The model and variable correlations remained unchanged with removal of this participant, $F(4, 86) = 1.187$, $p = 0.32$, adjusted $R^2 = 0.05$.

Table 4-2 Multiple linear regression to predict macular pigment optical density

(a) MPOD	B	95% CI for B		SE B	β	R^2	ΔR^2
		LL	UL				
Model						0.060	0.017
Constant	0.494	0.353	0.636	0.071			
Electronic Device Use	-0.002	-0.012	0.009	0.005	-0.034		
Dietary L/Z intake (screener)	0.000	0.000	0.001	0.000	0.150		
Age	-0.003	-0.005	0.000	0.001	-0.193		
Sex	-0.015	-0.087	0.058	0.036	-0.043		

(b) MPOD	B	95% CI for B		SE B	β	R^2	ΔR^2
		LL	UL				
Model						0.132	0.087
Constant	0.431	0.291	0.571	0.070			
Electronic Device Use	-0.003	-0.013	0.008	0.005	-0.052		
Plasma L/Z	0.332	0.123	0.541	0.105	0.364		
Age	-0.003	-0.005	0.000	0.001	-0.194		
Sex	0.028	-0.045	0.102	0.037	0.088		

(a) and (b) indicate different models. Abbreviations: B, unstandardized regression coefficient, β, standardized coefficient; CI, confidence interval; LL, lower limit; L/Z, lutein and zeaxanthin; MPOD macular pigment optical density; R^2, coefficient of determination; SE B, standard error of the coefficient; UL, upper limit; ΔR^2, adjusted R^2.

The multiple linear regression to predict MPOD from ED use, plasma L/Z, sex, and age was statistically significant, $F(4, 77) = 2.927$, $p = 0.026$, adjusted $R^2 = 0.087$ (Table 4-2, (b)). Statistically, plasma L/Z was the only variable that added significantly to the prediction ($p = 0.002$). Two assumptions were violated with one leverage value greater than 0.2 and one studentised residual value greater than three SD. The leverage value was from the same participant found in previous tests with the unusually high L/Z intake of 22.4 mg/day (600 mg/month) and high plasma L/Z concentration of 1.25 μg/mL. The other violation was from a participant without a high L/Z intake (22.83 mg/month) but a high MPOD value (0.87 ODU). The model and variable correlations strengthened slightly with removal of these participants, $F(4, 75) = 3.012$, $p = 0.23$, adjusted $R^2 = 0.092$.

4.4.3 Regression models to predict plasma lutein and zeaxanthin

A multiple linear regression to predict plasma L/Z from screener dietary L/Z intake, body fat percentage, sex, and age was statistically significant, $F(4, 81) = 23.16$, $p < 0.001$, adjusted $R^2 = 0.51$ (Table 4-3, (a)). Statistically, all four variables added significantly to the prediction. Once again, the participant with an unusually high L/Z intake of 22.4 mg/day violated all assumptions. The model and variable correlations weakened with removal of this participant, $F(4, 80) = 11.004$, $p < 0.001$, adjusted $R^2 = 0.323$. Age no longer significantly contributed to the prediction. With removal of this participant one assumption was violated with two leverage values greater than 0.2, these participants reported a high L/Z intake of greater than 400 mg/month. With removal of these participants the model and variable correlations weakened again but remained significant, $F(4,78) = 7.934$, $p < 0.001$, adjusted $R^2 = 0.253$

Table 4-3 Multiple linear regression to predict plasma lutein and zeaxanthin concentrations

(a) Plasma L/Z	B	95% CI for B		SE B	β	R^2	ΔR^2
		LL	UL				
Model						0.533	0.510
Constant	0.312	0.195	0.428	0.058			
Dietary L/Z intake (screener)	0.001	0.001	0.001	0.000	0.493		
Body fat percentage	-0.008	-0.011	-0.004	0.002	-0.398		
Sex	-0.150	-0.211	-0.090	0.030	-0.435		
Age	-0.003	0.000	-0.005	0.001	0.194		

(b) Plasma L/Z	B	95% CI for B		SE B	β	R^2	ΔR^2
		LL	UL				
Model						0.352	0.320
Constant	0.357	0.213	0.501	0.072			
Dietary L/Z intake (24DR)	0.009	0.002	0.017	0.004	0.228		
Body fat percentage	-0.008	-0.012	-0.004	0.002	-0.406		
Sex	-0.178	-0.248	-0.109	0.035	0.269		
Age	0.004	0.001	0.0060	0.001	-0.516		

(a) and (b) indicate different models. Abbreviations: B, unstandardized regression coefficient; β, standardized coefficient; CI, confidence interval; LL, lower limit; L/Z, lutein and zeaxanthin; MPOD macular pigment optical density; R^2, coefficient of determination; SE B, standard error of the coefficient; UL, upper limit; 24DR, 24-hour diet recall; ΔR^2, adjusted R^2.

A multiple linear regression to predict plasma L/Z from 24DR dietary L/Z intake, body fat percentage, sex, and age was statistically significant, $F(4, 81) = 11.00$, $p < 0.001$, adjusted $R^2 = 0.320$ (Table 4-3, (b)). One assumption was violated with one leverage value greater than 0.2, this participant was the same that violated assumptions in previous tests with the unusually high L/Z intake of 22.4 mg/day (600 mg / month) from the L/Z monthly screener and high plasma L/Z of 1.25 μg/mL. The model and variable correlations weakened with removal of this participant, F(4,

80) = 7.652, p <0.001, adjusted R^2 = 0.241. Dietary intake from 24DR did not remain a significant predictor of plasma L/Z concentrations (p = 0.23).

4.5 Discussion

This study investigated the association between MPOD, age, sex, daily electronic device use, dietary L/Z intake, and plasma L/Z concentrations in healthy Australian adults. The only variable MPOD was significantly correlated with was individual and combined plasma L and Z values. The models to predict MPOD indicated only plasma L/Z was a significant predictor (Table 4-2). This is the first study to the author groups' knowledge that investigated whether a relationship between usual ED use and MPOD status exists. The results indicate that blue light exposure is not presently related to MPOD status. Therefore, blue light exposure is not presently a risk factor for low MPOD which has been associated with risk of age-related macular degeneration. [53] This result is in agreeance with committees of experts with reported position statements on blue light exposure such as the ICNIRP. [176-178] Also, in alignment with committee conclusions, this outcome does not mean that continued monitoring and research into potential damage from light emitting diode blue light exposure is unwarranted.

The lack of relationship found between ED use, as a proxy for blue light exposure, and MPOD may be due to a number of reasons. One reason is that the exposure to blue light from ED in this population was not intense enough or long enough to result in levels of photochemical damage that were observable through MPOD status. [23, 181] This could mean that chronic ED use is safe in this population, or that the methods utilised were unable to detect negative outcomes on macular health. The aspects of the method that may have meant no relationship was detected were the low validity of the EDUQ, the measure selected as an indicator of macular health, sample size, and population demographics.

The EDUQ used to measure daily ED use behaviours has demonstrated low validity but was selected as it is the only published tool available (Chapter 3). Improvement of this tool or development of a more valid tool may benefit future investigations.

Measurement of MPOD through HFP is specific to concentrations of L/Z in the macula, however it may not be sensitive enough to show the impacts of ED blue light exposure. The reason MPOD may not be sensitive enough is that MPOD status would only become negatively impacted in the situation that L/Z are acting as direct antioxidants to combat reactive oxygen species generated as a result of BL exposure (photochemical damage); and L/Z macular concentrations are not being replaced at equal rates by circulating L/Z concentrations provided by dietary intake or other tissues where L/Z are found, such as adipose tissue. The population observed did not provide a diverse

enough array of behaviours and characteristics for such a relationship to be exposed. For example, only eight participants reported an average daily ED use less than 5 hours/day, two participants with mean daily L/Z intake below previously reported Australian average intake of 0.83 mg/day [13], and only 7 participants presented with an MPOD that expert panels have proposed as a low (<0.2 ODU). [230] It should be noted the population observed for mean Australian dietary intake, although a large sample size, was in adults 47 years or older and a more recent but smaller dataset of a population similar to that in this study indicates intake may be approximately 2.4 mg/day (Chapter 2). Future studies may benefit from a larger sample size and a goal for more participant diversity in MPOD, daily ED use, and dietary L/Z intake.

Another aspect of the method to consider is that a cohort study design may be needed to observe whether a relationship between ED blue light exposure and MPOD exists. Impacts of ED blue light on MPOD may be small and cumulative over the lifetime, thus it may be that the impacts are not yet be observable in this study population. For example, a participant that would be of interest to follow longitudinally is a 24-year-old female participant with a MPOD of 0.34 ODU, mean L/Z intake of 0.49 mg/day, plasma L/Z of 0.09 μg/mL, body fat percentage of 22.5% and mean daily ED use of 12.79 hours. The MPOD was over 0.20 ODU so not deemed low, however, with continued low dietary L/Z intake and high ED use it may hypothetically become lower over time. In addition to MPOD not being sensitive enough, only measuring MPOD may miss other proxy markers of macular health such as lipofuscin, drusen, basal laminar and linear deposit concentrations. Many of these other markers can be measured by optical coherence tomography. [231] Future studies may look to measure such markers in conjunction with ED use to investigate whether these markers of poorer macular health are increased in chronic high ED users.

The results of this study indicate that dietary L/Z intake was not a predictor of MPOD status, however plasma L/Z was. The association between MPOD and plasma L/Z of $r = 0.32$, $R^2 = 0.09$ ($p = 0.002$) was similar to that reported in previous studies. [55, 133, 232, 233] The confounding impact of adipose tissue is one reason plasma L/Z has been proposed to be not as strongly correlated with MPOD as might be expected for an objective measure. [134] Prior research has demonstrated an inverse relationship between MPOD or serum L/Z and both body mass index (BMI) and body fat percentage measured by dual-energy X-ray absorptiometry or bioelectrical impedance. [55, 133] The present study found a stronger inverse relationship between BMI and MPOD or plasma L/Z ($r = -0.37$, $p < 0.001$ and $r = -0.40$, $p < 0.001$ respectively) than that found in the study of 278 adults by Hammond et al. [133]. Conversely, the significant inverse relationship found between body fat percentage and MPOD or plasma L/Z found by Hammond et al. [133] was not found in the present study despite a similar distribution of population body fat percentages. In

the study by Nolan et al. [55] males and females were separated for analysis involving BMI or body fat percentage. Male BMI and body fat percentage were significantly inversely correlated with MPOD status, but not serum L or Z. Female BMI and body fat percentage were not significantly correlated with MPOD, but body fat percentage was significantly inversely correlated with serum Z (not L). In the present study, for males the only significant relationship was an inverse one between body fat percentage and MPOD ($r = -0.40$, $p = 0.028$) with a similar strength to that reported by Nolan et al. [55]. Female BMI and body fat percentage were significantly inversely correlated with plasma L/Z ($r = -0.22$, $p = 0.004$ and $r = -0.43$, $p < 0.001$ respectively). Lastly, only female BMI and not body fat percentage were significantly inversely correlated with MPOD, $r = -0.36$, $p = 0.003$. The relationships shown for BMI or body fat percentage with MPOD, and plasma L/Z are inconsistent. This inconsistency means it remains unclear how body fat levels influence circulating blood L/Z and MPOD status in males and females. Additionally, a measure of body fat rather than BMI is needed to explore this relationship further. In addition to using a measure of body fat, future research should also look to report on the weight history of participants. Changes to adiposity has been shown to influence blood L/Z concentrations. [136] Only 11% of participants in the present study reported they were attempting to lose weight at the time of data collection. Whether weight history impacted the heterogeneity in study outcomes related to adiposity, diet L/Z, plasma L/Z and MPOD cannot be determined as prior research have not reported on any indications of weight history or participant energy balance.

In this study MPOD was not significantly correlated with dietary L/Z intake. Prior research has reported mixed results, correlations have ranged from non-significant to moderate strength with significance such as $r = 0.48$ ($p < 0.01$) [232]. [55, 133, 233] Many prior studies measured dietary L/Z intake via a food frequency questionnaire with a 12-month recall timeframe. Interestingly, the strength of correlations observed between plasma L/Z and dietary L/Z in this study and the prior research is mixed. In this study, the association observed between plasma L/Z and dietary L/Z intake from the monthly screener was $r = 0.28$, $R^2 = 0.35$, $p = 0.008$. Prior research has reported correlation coefficients of 0.20 [233] up to 0.74 [232]. The variability in correlation strengths between dietary L/Z and plasma L/Z or MPOD are foreseeable due to the lack of tools available that have been validated to capture dietary L/Z intake. [67] The potential poor reliability of dietary L/Z intake data is highlighted in this study with the change to the model for predicting plasma L/Z when the monthly L/Z screener was replaced with the single 24-hour diet recall. The non-ubiquitous distribution of L/Z across foods and half-life of L/Z that is longer than 24-hours suggests a single 24-hour recall is unlikely to be representative of plasma L/Z. [120, 121, 138] The overall model to predict plasma L/Z concentrations when using the monthly L/Z screener was stronger than when

substituted with the 24DR. However, 24DR was a greater predicter within the model than the monthly L/Z screener was (Table 4-3). It should be noted this was with the very high L/Z diet intake and plasma participant included and once this participant was removed both models weakened markedly (Outlier participant characteristics: >600 mg/month from monthly L/Z screener, 14.7 mg from 24-hour diet recall, plasma L/Z 1.25 μg/mL).

Plasma L/Z was a greater predictor of MPOD than dietary L/Z intake was. While dietary L/Z was significantly correlated with plasma L/Z, it was weak and it would be plausible to expect a stronger relationship. However, a more valid dietary L/Z intake tool (Chapter 2) and greater understanding of interactions of blood L/Z with other bodily tissues is needed. An additional factor to be considered in future studies when attempting to relate dietary L/Z intake to plasma L/Z is the bioavailability of foods reported by participants. Weighting of foods by their bioavailability may assist in a reported value for dietary intake that is more closely aligned to levels present in the blood. [98] At present future studies should look to continue capturing both dietary L/Z intake and blood L/Z concentrations as their individual strength of relationship with MPOD continues to be inconsistent.

A strength of the present study was the capture of both dietary L/Z and plasma L/Z rather than one or the other. Another strength was the capture of body fat percentage rather than relying on BMI to make inferences about the interactions between body fat and plasma L/Z or MPOD. Limitations included the lack of diversity in ED use, sex, age, and educational status in the study population.

4.6 Conclusion

This study found that ED use, age, sex and dietary L/Z intake or plasma L/Z were not able to predict MPOD status in healthy adults that were predominantly young, female, and reporting a mean ED use of more than 9 hours/day. These outcomes indicate that ED use is not negatively related to macular health. However, the EDUQ tool has reported poor validity, and MPOD may not be an adequate indicator of macular health in this scenario. Further exploration of this relationship is warranted. Future studies may benefit from improving the validity of ED use capture, and including alternate measures of macular health such as drusen deposits. Plasma L/Z were the only variables individually correlated with MPOD in this population. Significant variables in the model to predict plasma L/Z concentrations were dietary L/Z intake, body fat percentage, age and sex. The outcomes of this study indicate that the relationship between MPOD, plasma L/Z, dietary L/Z intake and body fat percentage continue to be inconsistent, and refinement of dietary intake tool validity and physiological understanding of interactions between the observed variables is needed.

4.7 Summary

This study looked to apply the newly developed tools from Chapter 2 and 3 and addressed the third thesis objective, and barrier 2 (Figure 4-1). MPOD was not predicted by usual hours of ED use, dietary L/Z intake, age, or sex in Australian young adults. Blue light exposure from ED does not appear to be a confounding factor in the relationship between dietary L/Z and MPOD. The findings from this chapter confirm the role of habitual dietary L/Z intake in research relating to criteria 4 and 6, and inform directions for future research relating to these criteria.

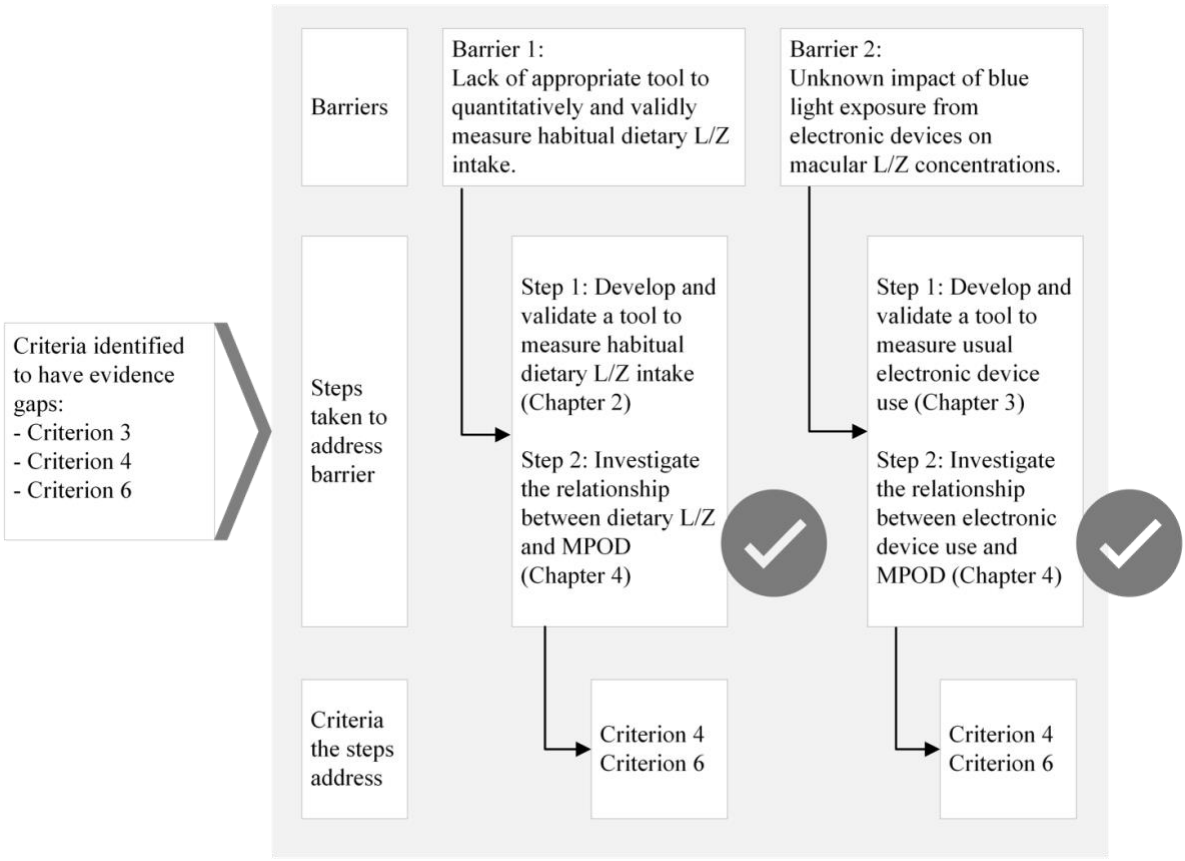

Figure 4-1 Steps addressed as part of Chapter 4 to improve the lutein and zeaxanthin evidence base related to the 9-criteria by Lupton et al. [2]

Chapter 5 Building food composition tables: Extraction methods to measure lutein and zeaxanthin concentrations in select Australia foods

This chapter reviews literature relevant to the variation and analysis in food L/Z concentrations (section 5.1). The literature explores pre- and post-harvest factors impacting food L/Z concentrations, food L/Z sampling and analysis methods, and the current status of US, UK and Australian food composition data. Additionally, this chapter then describes the application of this review of the literature though presenting the results of the original research study addressing thesis objective 4 (section 5.2 – 5.7), the investigation of an appropriate extraction method for analysing food L and Z concentrations suitable for building local Australian FCT.

5.1 Review of food composition data in relation to dietary lutein and zeaxanthin intake

The availability and use of food composition data relates directly to criteria 2 and 3 of the thesis research framework (Figure 1-3, page 61). Criterion 2 is a reliable analysis method and criterion 3 is a food database with known amounts of the bioactive constituent. Criterion 2 applies to reliable analysis methods of foods but also other analyses needed such as blood L/Z concentrations. Criteria 2 and 3 in this section will be explored in relation to food analysis methods. Numerous factors must be considered when selecting the most appropriate food composition data to calculate L/Z reported from dietary intake measurements. These factors include the selection of representative food samples and analytical methods used to measure food L/Z concentrations. A commonly used analytical method to measure food L/Z concentrations is high performance liquid chromatography (HPLC). Optimal methods to measure carotenoids such as L/Z have changed over time and may be different between foods. [234] Selection of representative food samples is important as concentrations of L/Z within a food may be influenced by the food variety, growing conditions, supply chain conditions, and processing before consumption. Having enough food composition data (criterion 3) and appropriate food composition data to reference, has the potential to impact research outcomes of other criteria such as 4–8. Research to determine an amount of dietary L/Z that appears to positively influence MPOD and/or risk of conditions such as AMD rely heavily on the data used to determine dietary L/Z. Thus, ensuring that a method to measure dietary intake of L/Z is valid involves understanding the status of food composition data.

5.1.1 The food variety in the selection of representative data

To measure dietary L/Z intake of a population, the food composition data being referenced must be representative of the foods consumed by the populations. [4, 235] One aspect of a representative food sample is consideration of the food variety. The concentration of carotenoids such as L/Z can

be different between food varieties. In non-plant food such as eggs, the L/Z concentration is influenced by the feed provided to the chickens. [57] Therefore, concentrations have potential for high variability between brands and farms. The concentration of L/Z both within and between plant types is also highly variable, in part due to genetic differences. [168] The cultivar of a plant determines its ability to make and store carotenoids. The cultivar describes small, heritable differences within a single species. For example, grown in the same field, L concentrations within six varieties of broccoli has been reported to range between 0.41 and 1.02 mg / 100 g. [236] In green fruits and vegetables, carotenoids are located in the chloroplasts of plant cells, bound to light-harvesting photosystems 1 and 2. [147, 168] Within the chloroplasts, the types and ratio of carotenoids is fairly constant, with L often making up 40–50% of total carotenoids, and Z undetectable or only found in small concentrations. [168] Similar to green varieties, in orange, yellow and red fruits, carotenoids can be found in chloroplasts. However, the location and concentration of carotenoids changes during the ripening process of many of these fruits. This change is due to the degradation of chloroplasts, and development of chromoplasts (another sub-cellular organelle). Carotenoid types subsequently accumulated in the chromoplasts. The accumulation is determined by the presence and activity of specific ripening genes. [168] For L/Z synthesis to occur the activity of genes to produce several enzymes are required; lycopene ε-cyclase, lycopene β-cyclase, carotene hydroxylase enzymes which include ε- and β-ring hydroxylases. [168, 225] The wide genetic variability possible therefore makes plant cultivar an important factor when determining whether data is representative of food L/Z concentrations. To most accurately capture dietary L/Z intake, food composition data that has measured the variety of food cultivars consumed by the population of interest is needed. Additionally, the differences in carotenoid storage between plant species present challenges when attempting to optimise extraction and analysis methods (section 5.1.4).

5.1.2 Pre-harvest factors in the selection of representative data

Plant carotenoid concentrations are also influenced by multiple pre-harvest factors. Representative food composition data would ideally capture the variability in food L/Z concentrations these pre-harvest factors can contribute to. The pre-harvest factors that can influence L/Z production in plants include plant cultivar, climate, soil, and maturity at harvest. [169, 170] Plant cultivar has already been previously described (section 2.1.5.1). Climatic variation in open fields and greenhouses, such as temperature and sunlight exposure, can influence L/Z concentrations. Low sunlight (low ultraviolet radiation) and temperature may result in decreased rates of carotenogenesis. Carotenogenesis is the production of carotenoids, including L/Z. Conversely, excessive temperatures and high sunlight can promote photodegradation of L/Z, and down regulation of

carotenogenesis. The impacts of climate have been demonstrated with kale grown under polyethylene roofing. Polyethylene roofing can filter the intensity of sunlight reaching plants. [170] Concentrations of L from kale grown under polyethylene roofing were reported to be higher in summer compared to winter. This may be due to optimal protection from sunlight in summer, but too little sun in winter. Similarly, green leafy vegetables grown in open fields have shown higher concentrations of L and other carotenoids in winter compared to summer. [170] Lastly, maturity at time of harvest can influence L/Z concentrations. Biosynthesis of L/Z is enhanced in most fruits and vegetables that are approaching maturity or ripeness. For example, L concentrations in mature fully expanded kale leaves were higher compared to younger leaves. Notably, leaves reaching senescence were reported with the lowest concentrations. [170, 237] Climate and maturity at harvest impact food L/Z concentrations. Therefore, when attempting to select representative food composition data, capture of variability from pre-harvest factors for foods relevant to the population of interest is needed.

5.1.3 Post-harvest factors in the selection of representative data

Post-harvest factors that affect L/Z plant concentrations are storage conditions and processing methods. Ideally, representative food composition data would capture the variability in food L/Z concentrations these post-harvest factors can contribute to. Losses of L/Z during storage and processing can occur via three mechanisms: isomerisation, enzymatic oxidation and thermal oxidation. Interestingly, even after being detached from the main plant body, plants remain active and responsive to environmental stimuli. Thus, storage conditions such as lighting intensity and changes, temperature, duration in storage and atmosphere (oxygen and carbon dioxide concentrations) may be responsible for fluctuations in L/Z concentrations. [170, 238] Common food processing methods include blanching, boiling, steaming, frying, baking, grilling, chopping, and juicing. A review investigating post-harvest effects on food carotenoid concentration reported variation in L/Z losses with processing before consumption. [170] After blanching, boiling, or steaming changes in concentrations including total losses, minimal change, and increases have all been reported amongst a variety of foods. Frying, baking, and grilling were recorded to cause thermal oxidation, lowering L/Z concentrations. Chopping, and juicing expose food tissue to oxygen and light, resulting in concentration decreases from enzymatic oxidation. [170] Therefore, prior to consumption of L/Z containing foods, a wide array of factors will influence the concentration of L/Z present at the time of consumption. Usual storage and processing steps before food consumption in the population of interest must be considered to obtain food composition data representative for use in capturing dietary L/Z intake.

5.1.4 Quantification of lutein and zeaxanthin in foods

The accuracy of the methods used to quantify L/Z in foods can impact the accuracy of subsequent applications of the data such as estimating milligrams of L/Z intake from a diet record. There is no universal method for quantification of food carotenoids due to variation in method needed for different research aims and food macronutrient composition. [234, 239, 240] Method variations are required due to differences in food matrices, such as the presence of chlorophyll or high fat concentrations. [241] Alternatively, the aims of an investigation may impact which method is most appropriate. For example, aims to measure the profile of multiple carotenoids within a food may need a different method variation compared with a focus on a single carotenoid such as L or Z. However, there are common factors that must be considered when analysing food for carotenoid concentrations. These factors include representative food sampling, food processing methods, extraction solvents and processes, and the analytical method.

The importance of representative food sampling has been described earlier in the context of food composition data selection for use in calculating dietary intake (section 5.1.1 to 5.1.3). To build relevant food composition tables (FCT), representative sampling must occur at the analytical stage. Heterogeneity in L/Z concentrations can stem from cultivar variation and pre- and post-harvest factors. This heterogeneity demands multiplicity of food samples analysed, rather than single sample analysis that has been described a common analytical error. [240] A selection of multiple food samples that are representative of what the population of interest consumes is necessary.

The food processing methods prior to extraction can influence the outcomes of L/Z food analysis. [240] The factors of food processing for consideration include milling or cooking processes, the part of the food sample chosen for processing, and storage time before analysis. In the context of a FCT, it is important to consider the form in which a food is ingested by the consumer. In the case of foods such as grains, analysis after usual milling processes may be most appropriate. Alternatively, with foods such as pumpkin, cooking processes like steaming, boiling, or baking may be needed to ensure it is representative of population consumption. Further to this, the part of a food analysed is important to consider. The distribution of carotenoids within food has been shown to be heterogeneous. [242-244] Selection of multiple food units for analysis of foods that come in a bunch or group has shown to be important. In a single bunch of bananas, carotenoid content was shown to be different between individual bananas. [244] Additionally, within a single unit of food, carotenoid content is variable between different parts of the food, e.g. top and bottom. [242, 243] Observation of sweet-corn cobs indicated that the kernels at the bottom of the cob contained significantly less, 12% to 17%, L and Z compared to kernels at the top of the cob. [242] Another

food processing factor for consideration is sample storage time before analysis. As previously discussed (section 2.1.5.3), storage time and conditions can influence L/Z food concentrations. [170, 238] Logically, when performing analysis it is recommended that samples are analysed as soon as possible after collection. [240] A method commonly used to preserve samples that need to be stored before analysis is lyophilization (freeze-drying). Lyophilization has been shown to reduced carotenoid loss during low temperature storage compared with food pulp storage. [245, 246] A downside of choosing to lyophilize a sample is increased sample porosity which can increase oxygen exposure. [247] Increased oxygen exposure may increase carotenoid degradation. Additionally, there can be error associated with returning analysed values to the equivalent fresh weight of the food. The error with this calculation lies with use of proximate analysis to determination the food moisture content. [240] Selection of food processing steps most representative of the state and storage time a food will be at when ingestion occurs must be considered when analysing food L/Z concentrations for FCT data.

The selection of extraction method processes and solvents are other important factors that may influence L/Z concentrations measured in foods. The method process includes length of time the extraction takes, light exposure and temperature exposure. Longer extraction times may increase risk of carotenoid isomerisation and degradation through increased opportunity for exposure to light and oxygen. [248] Exposure of a sample to light increases the rate of photodegradation and isomerisation of carotenoids. [248] Filtering out the light wavelengths that impact carotenoids has been shown to slow the sensitisation of a sample to photoisomerization. [249] Attempting to keep extraction times short, and exposure to oxygen and heat to a minimum are method processes for consideration. Another method process to consider is the use of saponification or sonication. Saponification may be a worthwhile step as it hydrolyses carotenoid esters and removes lipids and chlorophylls which are not needed. L/Z can exist in both free and esterified forms within a food. [242] However, saponification may also result in the destruction or isomerisation of carotenoids within foods. [234, 250, 251] Sonication has also been utilised during extraction due to the potential for mechanical forces (rather than chemical) to aid release of carotenoids from the plants structures. For example, mechanical force disrupting cell wall structures and allowing for release of carotenoids into solution for a solvent extraction. [234] Solvent choice is another important consideration. Commonly used solvents include acetone, hexane, petroleum ether, methanol, ethanol, dichloromethane, and tetrahydrofuran. Acetone has been used in validated methods as it dissolves both carotenes and xanthophylls. [252] Hexane and petroleum ether are reported to efficiently dissolve carotenes but not xanthophylls. Conversely, methanol and ethanol dissolve xanthophylls efficiently but not carotenes. [234] The differing properties of solvents has meant

utilising mixtures of solvents to test for optimal methods is important. [253-255] The differences in food matrices and L/Z concentrations mean the optimal extraction method can vary between foods. [234] The extraction methods used in the analysis of L/Z foods for a FCT should form part of the decision of whether the data is representative for the application of interest.

The final consideration in quantifying food L/Z concentrations is the analytical method of choice. Analytical methods that have been used to observe food carotenoid concentrations include HPLC with photodiode array detection (DAD), HPLC with mass spectrometry, supercritical fluid extraction with carbon dioxide, and resonance Raman spectroscopy. [240] There is increasing use of HPLC-mass spectrometry in combination with atmospheric pressure chemical ionisation, electrospray interface, or off-line NMR to assist determining the geometrical isomers of carotenoids present. [256-258] However, to date, HPLC with DAD method has been the most commonly used. The DAD provides the visible absorption spectra of the carotenoids. The column selected is important for HPLC as a method for L/Z analysis. The use C_{18} reverse-phase HPLC columns is commonly used to investigate concentrations of carotenes and other carotenoid end groups. However, L and Z are polar oxygenated carotenoids and are often only able to be partially separated with this column type. The use of a C_{30} silica-bonded column and normal-phase HPLC is able to separate L and Z effectively. [259] To determine individual concentrations of L and Z in foods for use in FCTs the latter HPLC method is an appropriate choice.

Representative food sampling, food processing methods, extraction solvents and processes, and the analytical method form part of determining if the analysis of food L/Z concentrations has been conducted appropriately and reliably. The differences that exist between foods demand that a method requires optimisation to the food of interest. [234] The impact of pre- and post-harvest factors, food sampling methods and analytical methods indicate that L/Z concentrations may be highly variable in different foods supplies both within and between countries. A FCT that is specific to the location or population of interest is important to develop to meet the third criterion discussed by Ranard et al.[1] (Figure 1-1, page 31). The methods for sampling and analysis that have been used to build a FCT inform whether the table is appropriate for use in dietary L/Z intake measurement.

5.1.5 Food composition tables

Three large databases that report L/Z are, the USDA FCTs, the McCance and Widdowson's Composition of Foods Integrated Dataset (CoFID) and the Food and Nutrition Australia New Zealand (FSANZ) food and nutrient database. The FSANZ database contains 26 entries for L of

local foods such as tomato, butter, cheese, carrot, egg, and broccoli. [229] These 26 entries were from samples collected as part of the 2014-15 or 2018-19 key foods analytical program. Further detail on the extraction methods used in this program is not available. A composite sample or individual testing of eight food purchases from five Australian states was tested. [158, 260] The CoFID published in 2010 also only reports L concentrations but reports over 200 values.[171] Samples such as fruits and vegetables were collected over a number of seasons and up to 22 food purchases analysed as a composite sample were analysed. [261] The analysis process for the carotenoids in this dataset involved analysis by a lab accredited by the United Kingdom Accreditation Service. The method was focussed on Vitamin A and E extraction (not L/Z) and involved a saponification step, solvent extraction and HPLC analysis. [261] The FCTs for the USDA is the largest dataset, and L/Z are predominantly reported as a combined value. [138] The USDA FCTs are updated regularly and contain a mixture of both analytical and proximate data. Analytical data for L/Z may be recently measured such as 2021, or over 20 years old. The proximate data is an estimated value for L/Z using pre-determined assumptions, such as a percentage loss of L/Z with cooking for a food. [138]

Table 5-1 Lutein and zeaxanthin concentration in seven commonly consumed foods between USDA, FSANZ and CoFID food composition tables

Food	Dataset and L/Z Concentration (μg/100g)		
	USDA (L/Z combined)	CoFID (L only)	FSANZ (L only)
Asparagus	771	1450	DNR
Broccoli	1403	DNR	352.5
Egg, cooked, hard-boiled	353	97	342
Kiwifruit	122	161	120
Peas, green	2400	1134	620
Spinach, baby	6020	5782	DNR
Tomato, raw	123	108	18.5

Abbreviations: L, lutein; Z, zeaxanthin; USDA, United States Department of Agriculture [138]; CoFID, Composition of Foods Integrated Dataset [171]; FSANZ, Food Standards Australia New Zealand [229]; DNR, did not report.

The FSANZ and CoFID database are not large enough to comprehensively analyse for habitual L/Z dietary intake from an Australian or UK resident respectively. Thus, the USDA database is the best option available to estimate dietary L/Z intake, even in a UK or Australian resident. However, in using the USDA database, it is essential to consider between country differences in measured L or Z concentrations. As seen in Table 5-1, reported concentrations of L/Z within individual foods vary between these FCTs. [138, 171, 229] Variations may be due to both agricultural and preparation

methodology for analysis (section 5.1.1 – 5.1.4). [169, 170] The example of differences present between the three databases in Table 5-1 indicate that if dietary L/Z intake were to be estimated using the USDA in an Australian population, misestimation due to differences in the FCT values would occur. For example, a difference of 1000 μg/100 g of broccoli. These value differences likely impact outcomes of studies investigating dietary L/Z intake in non-US populations. Therefore, investigation into the extent of differences between the USDA FCT and non-US food supplies is warranted to determine whether development of FCTs local to a non-US population of interest is justified. The extraction methods used in the FSANZ database are unavailable, and the USDA and CoFID methods are outdated or have not been specific to L/Z analysis. [138, 229, 261] Therefore a first step in investigating food supply differences is determining an extraction method specific to the food of interest that maximally capture L/Z concentrations for use in FCTs. The preliminary outcomes of this analysis can then be used to indicate whether differences between the USDA and Australian FCTs exist (section 5.6.2).

5.2. Publication details

Section 5.3 to 5.7 of Chapter 5 includes the manuscript published the International Journal of Food Science and Technology (Journal Impact Factor: 3.3; Quartile 2). Numbering of tables, figures, and references are presented as part of the whole thesis and as such numbering is different to that of the submitted work. Graphical representation of data from Tables 5-3 to 5-6 were not part of the submitted manuscript but are presented in Appendix E-5. No other text in section 5.3 to 5.7 is different to the submitted manuscript.

N. K. Fitzpatrick, V. Chachay, A. Shore, S. Jackman, S. Capra, J. Bowtell, D. Briskey. Building food composition tables: extraction methods to measure lutein and zeaxanthin concentrations in select Australian foods. International Journal of Food Science & Technology. 2024. doi: 10.111/ijfs.16938

5.3 Introduction

Quantification of constituents from dietary intake, and their subsequent implication in prevention and management of non-communicable diseases, is reliant upon food composition tables (FCT) [2]. To effectively investigate relationships between dietary intake and disease, data within a FCT must be from reliable and representative analysis methods, and contain enough data points to adequately capture dietary intake.

Lutein and zeaxanthin are two dietary carotenoids that have been investigated for their relationship in reducing risk and severity of age-related macular degeneration [149]. Many countries do not have

comprehensive FCTs for lutein and zeaxanthin, one exception is the United States Department of Agriculture (USDA) tables [138]. In countries without comprehensive tables, such as Australia, attempts to capture dietary lutein and zeaxanthin intake have relied upon the USDA tables [13]. The Food Standards Australia and New Zealand (FSANZ) FCTs are not comprehensive with only 26 entries for lutein (not zeaxanthin) [229]. Comparison of the USDA and FSANZ tables suggest differences in food supply lutein and zeaxanthin concentrations may exist. Of five foods reported in both the FSANZ and USDA tables, including broccoli and green peas, two foods reported similar concentrations and three indicated differences of more than 250% [138, 229]. Differences between the tables may be related to factors including extraction and analysis methods, food sampling and preparation methods, food ripeness, and natural variation in concentration between food cultivars [168, 170, 240, 262]. Understanding of the factors that contribute to differences between the USDA and FSANZ tables is necessary to determine if the USDA tables are appropriate for use in an Australian setting. Extraction and analysis methodologies are two such factors. There are frequently used reliable methods to analyse food lutein and zeaxanthin concentrations, such as High Performance Liquid Chromatography with Photodiode Array Detection (HPLC-DAD) [240, 262]. There is no single extraction method that is most appropriate for all foods. Different methods to extract lutein and zeaxanthin have varying efficiency for different foods [234, 239, 240]. An extraction method specific to the substance and food of interest is important to ensure maximal capture of both free and esterified lutein and zeaxanthin in food samples [263]. Therefore, optimising an extraction method to improve assay efficiency is important [234]. The continued improvements to extraction and analysis methods for food lutein and zeaxanthin suggests existing values in FCTs may not be representative of the food supply [225]. For example, many of the entries in the USDA tables were not extracted and analysed using recent or lutein- and zeaxanthin-specific techniques [138]. In particular, lutein and zeaxanthin are predominantly reported as a combined value, rather than individually like is possible with more recent methods. For the few FSANZ entries, the commercial nature of the analyses conducted means details of extraction methods are unavailable, and therefore comparability of methods is limited [264].

The absence of a FCT that is accurate and specific to the population of interest, such as in Australia, has multiple implications. Not least that the reported intake values and strength of the relationship between dietary lutein and zeaxanthin intake and conditions such as age-related macular degeneration must be interpreted with caution [67]. Ideally, comprehensive Australian FCTs would be available for lutein and zeaxanthin analysed with methods optimal to the food and constituents of interest. Therefore, the aim of this study was to investigate optimal extraction methods for analysis of lutein and zeaxanthin in a select group of Australian foods analysed by HPLC-DAD for application in building FCTs.

5.4 Materials and methods

5.4.1 Chemicals

Acetone, ethanol, hexane, dichloromethane, methanol, acetonitrile, triethylamine analytical grade (sourced from Merck Chemicals, Australia). A reference lutein standard was purchased from Merck Chemicals Australia and used for quantification of a pure lutein product donated in kind by Pharmako Biotechnologies Pty Ltd, Sydney, NSW to be used for ongoing quantification. A reference zeaxanthin standard was donated in kind by the Queensland Alliance for Agriculture and Food Innovation.

5.4.2 Food sample collection

Foods selected for analysis were those available for purchase in Brisbane (Australia) from January 2020 to July 2021 and reported to contain above 100 µg/100g of lutein and zeaxanthin as per data from the USDA or FSANZ FCT [138, 229]. Foods reported to contain more than 100 µg/100g of lutein and zeaxanthin were selected to ensure high applicability to subsequent research on dietary lutein and zeaxanthin intake [67]. Foods selected for analysis were: broccoli (Brassica oleracea var. italica), broccolini (Brassica oleracea), baby orange capsicum (Capsicum annuum L.), baby spinach (Spinacia oleracea), and dried goji berry (Lycium barbarum). All food samples were grown in Australia except for dried goji berries grown in China, see Appendix E-6. The guideline document Generating Data for Food Standards Australia New Zealand Nutrient Databases (2019) and the Food Composition Data book by Greenfield and Southgate (2003) were used to inform the sampling strategy and volume of food for purchase [265, 266]. Convenience sampling was utilised for sourcing food samples from various venues (Woolworths, Coles, Aldi, independent grocers, and marketplaces) in Brisbane (Queensland, Australia), and included different origins of growth/harvest (Queensland and interstate). Enough units (e.g. one head of broccoli) were purchased such that the weight of the sample was a minimum 150g, or a volume (e.g. baby spinach) of two metric cups. Purchased samples were transported in cool conditions and stored in a refrigerator for no more than 1 day before undergoing lutein and zeaxanthin extraction. Each food type was denoted by a different number, and each different sample of a food purchased was denoted by a different letter (Table 5-2).

Table 5-2 Letter key for food samples

Number = Food	Letter per sample *, †	Example of food and sample together
1 = baby spinach	A = sample A	1A = sample A of baby spinach
2 = broccoli	B = sample B	
3 = broccolini	C = sample C	1B = sample B of baby spinach
4 = baby orange capsicum	D = sample D	
5 = dried goji berry		2A = sample A of broccoli

*Samples differ by their date or store purchased from. † Letters to denote different samples continue alphabetically with increasing numbers of samples

5.4.3 Food sample preparation

The shape and type of a food sample determined the preparation to obtain a 'reduced sample' [266]. A reduced sample refers to a representative part of the whole food. Variations in sample preparation included whether there was an inedible portion to be removed, or cooking process to be performed (e.g. steaming, boiling, frying). Sample preparation was performed so the sample analysed was representative of general population consumption [266]. The inedible portions removed were the bottom 2 cm of the broccoli stem, bottom 1 cm of the broccolini stems, and the seeds and stem of the baby orange capsicum. Broccoli and broccolini were cooked, steaming in a 1000W microwave until easily pierceable by knife point. The steamer was a standard household microwave safe plastic steaming container in which the container separates the food from water on the bottom of the container. The steaming time was 2.5min for broccoli, and 2min for broccolini. The foods were then chopped coarsely, mixed, and separated into quarters. One quarter was randomly selected and blended.

To achieve a homogenous consistency of the reduced sample there were two blending steps. The first blending step was homogenisation using a hand-held blender (Bamix® Mono blender 140W). Four of the five foods required the addition of distilled water to facilitate blending and achieve an even consistency. To determine the minimum volume of water required for these four foods, 0.25mL of distilled water per 1g of food was added and blending attempted. If blending was still unsuccessful, the ratio of distilled water to reduced sample was increased in 0.05mL increments until blending was successful. The volume of distilled water added per 1g of food was 1mL for broccoli, 1mL for broccolini, 0.7mL for baby spinach, and 1.5mL for dried goji berry. Approximately 2g of the blended food mixture was transferred to a 5mL vial, and 2mL of distilled water was added. The blended sample then underwent the second blending step and was homogenised using Kinematic Handheld Homogeniser POLYTRON® until a uniform texture was reached. A uniform texture was determined through visual observation and a degree of liquidity of the sample that would allow for pipetting with a 100-1000µL pipette tip.

5.4.4 Lutein and zeaxanthin extraction

Analytical methods described by Chandra-Hoie et al. (2017) [267] and Fanning et al. (2010) [225] were used as the initial reference extraction methods. Briefly, 200µL of prepared food sample and 400µL of acetone was added to a 1.5mL microfuge tube and mixed for 10s. To the resulting solution, 600µL of n-hexane was added, mixed for 10s then centrifuged for 4 min at 12,000rpm (or 17,709*g* force, Mikro 200 Hettich Zentrifugen). The supernatant was transferred to a glass culture tube and dried under nitrogen on a 39 °C hotplate until dry. The sample was reconstituted with 100µL of mobile phase (methanol 49.96%, acetonitrile 49.96%, triethylamine 0.08%), mixed for 10s and transferred to an amber HPLC vial for analysis.

Up to an additional eleven variations of the lutein and zeaxanthin extraction method were tested to determine variability in extraction efficiency. The extraction variations are outlined in Figure 5-1. Two variations occurred during the food sample preparation. The first was addition of 2mL of ethanol instead of distilled water before homogenisation. The second was after homogenisation where the food sample was sonicated at 4 °C for 30s (Qsonica Sonicators, Model CL-188). All other variations occurred after 200µL of the homogenised food sample was pipetted into a microfuge tube. The variations included: no addition of acetone, use of 80:20 hexane/dichloromethane (DCM) instead of hexane alone [263], saponification of the sample, and two extractions of hexane or hexane/DCM rather than one. Saponification was achieved by addition of 150µL of 10N potassium hydroxide (KOH) and incubated in water at 45 °C for 30min, or addition of 300µL of methanol sodium hydroxide (MeOH NaOH) and incubated in water at 60 °C for 30min.

5.4.5 Lutein and zeaxanthin analysis

Quantification of lutein and zeaxanthin was conducted using a HPLC system (Shimazdu, Kyoto, Japan) with DAD (SPD-M10Avp). Ten microliters of extract were eluted onto a Develosil 5µm RP-aqueous C30 140A, 250 × 4.6mm column with isocratic mobile phase containing methanol (49.96%), acetonitrile (49.96%), and 0.08% triethylamine at a flow rate of 1.2mL/min with a 30min run time [268, 269]. Detection of lutein and zeaxanthin was performed at 445nm [225, 226].

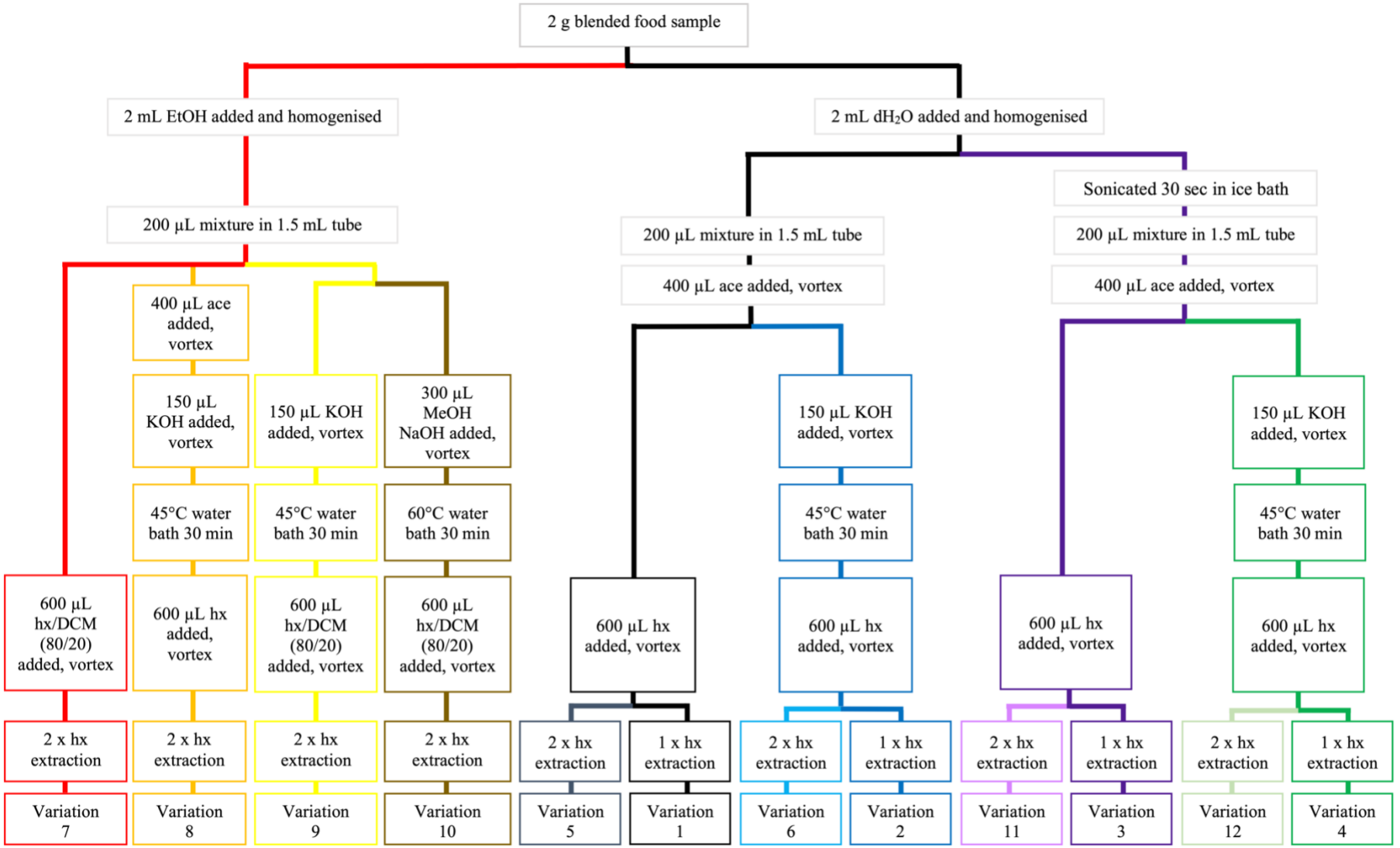

Figure 5-1 Variations to food preparation and extraction method.
Abbreviations: ace, acetone; DCM, dichloromethane; dH2O, distilled water; EtOH, ethanol; hx, hexane; KOH, potassium hydroxide; MeOH NaOH, methanol sodium hydroxide

5.4.6 Identification and quantification of lutein and zeaxanthin

Identification of lutein and zeaxanthin was conducted by comparison with the retention time and absorption spectra of the corresponding analytical standards. To confirm the purity and concentration of both lutein and zeaxanthin analytical standards, spectrophotometric absorbance of the analytical standards was performed, and peaks were established by HPLC-DAD. Concentration by spectrophotometric absorbance of lutein and zeaxanthin dissolved in ethanol was calculated by the following equation (1):

$$\text{Concentration} = \text{absorbance} / (\text{cuvette length} \times \text{extinction coefficient}) \quad (1)$$

Absorbance was measured at 445nm for lutein and 450nm for zeaxanthin. The length of the cuvette was 1 cm. The extinction coefficient (ε) used for lutein was 145 and zeaxanthin 141 [227]. The limit of detection at 445 nm for lutein was 0.009 and 0.05µg/mL for zeaxanthin. Standard curves measured for lutein were linear between the range of 0.009–90µg/mL with r^2 values of >0.99. Standard curves measured for zeaxanthin were linear between the range of 0.05–15 µg/mL with r^2 values of >0.99.

Method of standard addition determined assay return. Three 200µL food samples were spiked with 100µL of 90µg/mL lutein standard. The area under the curve of the concentration of lutein present before spiking was subtracted from the lutein spiked food samples. The remaining area under the curve value was compared to the area under the curve measured by the 90µg/mL lutein standard to obtain a percentage of lutein standard present in the spiked food sample.

5.4.7 Statistical analyses

The statistical software used was GraphPad Prism version 9.0.0. The differences in lutein and zeaxanthin concentrations when two extraction variations for a food were analysed were tested by Mann–Whitney two-tailed test, or by two-tailed paired t-test of the mean lutein and zeaxanthin concentrations from multiple food samples. Differences between three or more extraction methods from the same sample of food were tested using relevant one-way ANOVA and multiple comparisons or Kruskal–Wallis test and Dunn's multiple comparison. A statistically significant difference was set at $p < 0.05$. Measured concentrations of lutein and zeaxanthin are reported as mean µg/100g edible raw food portion for baby orange capsicum, goji berry and baby spinach, and mean µg/100g edible cooked food portion for broccoli and broccolini.

5.5 Results

A lutein and zeaxanthin value was detectable in all samples of all foods except for zeaxanthin in steamed broccoli, and for lutein in one sample of dried goji berries. A chromatogram depicting lutein and zeaxanthin of baby orange capsicum is shown in Figure 5-2.

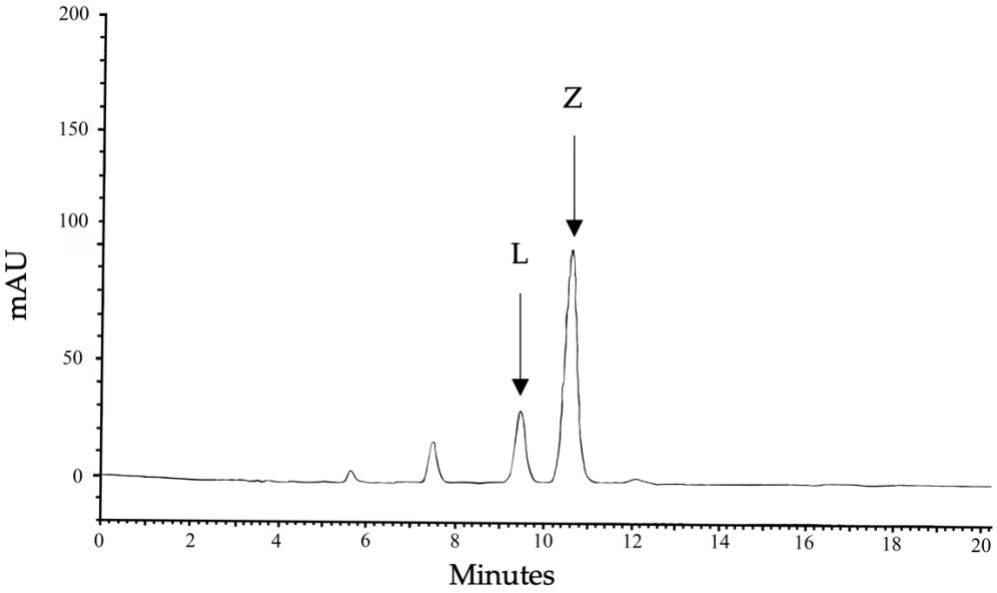

Figure 5-2 Capsicum, orange, baby chromatogram
Abbreviations: L, lutein; mAU, milli absorbance units; Z, zeaxanthin

5.5.1 Impact of extraction method variations on baby spinach

The process for determining whether a change in extraction method impacted measured lutein and zeaxanthin concentrations was performed incrementally. Variations that differed by a step in the extraction method were grouped together for comparison. For example, variation 1 and 3 were compared for the impact of a sonication step. Variations 1 and 2 were compared for the impact of a saponification step. Then variations 1 and 4 were compared for the impact of a sonication and saponification step (Table 5-3). Refer to Figure 5-1 for differences present in extraction steps. Baby spinach was selected as an example throughout the results section to demonstrate the incremental process of comparing the method variations. For the results of method variations comparison for broccoli see Appendix E-1, broccolini see Appendix E-2, baby orange capsicum see Appendix E-3, and dried goji berry see Appendix E-4. 5.5.1.1 Comparison of method variations 1, 2, 3, and 4 Differences between the method variations were tested with a Brown-Forsythe and Dunnett's T3 multiple comparisons test for comparing the mean lutein, and Kruskal–Wallis test and Dunn's multiple comparison test for comparing the mean zeaxanthin between the four variations (Table 5-3). The lutein ANOVA outcome was significant ($p = 0.003$), and the lutein concentration from variation 1 was significantly greater than variation 2 ($p = 0.01$). The zeaxanthin Kruskal–Wallis outcome was significant ($p = 0.007$), and the zeaxanthin concentration from variation 1 was significantly greater than variation 4 ($p = 0.008$). No other significant differences in lutein and zeaxanthin concentrations between method variations were present (see Appendix E-5, Figure 9-1 for graphical representation of data). The method recoveries for variations 1, 2, 3, and 4 measured by method of standard addition were not significantly different, and were 64%, 61%, 58%, and 60%

respectively. Of variations 1–4, variation 1 appeared the best to use, as the measured lutein and zeaxanthin concentrations were higher and/or the method was more time efficient to complete than variations 2, 3 and 4.

Table 5-3 Baby spinach, comparison of method variations 1, 2, 3, and 4

Sample ID	Lutein or zeaxanthin	Method variation (µg/100g)			
		1 [a]	2	3	4
1A (n 3) *	Lutein	8,301 ± 568	6,791 ± 254	-	-
	Zeaxanthin	259 ± 29	304 ± 24	-	-
1B (n 3)	Lutein	7,128 ± 197	6,194 ± 228	6,947 ± 158	6,455 ± 512
	Zeaxanthin	266 ± 5	190 ± 8	262 ± 21	191 ± 16
1C (n 3)	Lutein	6,842 ± 168	6,261 ± 240	6,897 ± 132	6,025 ± 382
	Zeaxanthin	224 ± 17	166 ± 9	196 ± 8	157 ± 11
1D (n 2)	Lutein	8,657 ± 2	6,914 ± 1576	7,231 ± 138	7,794 ± 577
	Zeaxanthin	303 ± 47	181 ± 55	264 ± 10	207 ± 9

[a] All samples combined (A, B, C, D) Variation 1 significantly different to variation ($P \leq 0.01$) * Variation 3 and 4 not completed for Sample A. Data presented as mean ± standard deviation. Differences between variations for L tested by Brown-Forsythe ANOVA and Dunnett's T3 multiple comparisons, and Kruskal-Wallis and Dunn's multiple comparisons for Z. Abbreviations: n, number of replicates analysed per sample

5.5.1.2 Testing of multiple hexane extractions

Given the moderate efficiency found from method variations 1–4, multiple hexane extractions were tested to improve on the moderate efficiency found from method variations 1 to 4 (Tables 5-4 and 5-5). Method variation 5 was different to variation 1 with two hexane extractions rather than one, and was conducted on Sample E (Table 5-4 and see Appendix E-5, Figure 9-2 for graphical representation). The two hexane extractions were analysed individually in addition to another two individually analysed hexane extractions (four total). Of the total lutein measured in the four extractions, extractions one to four returned a mean of 51%, 47%, 1.3%, and no detectable lutein respectively. Of the total zeaxanthin measured in the four extractions, extractions one to four returned a mean of 58%, 42%, and no detectable zeaxanthin respectively. The second hexane extraction increased the total lutein and zeaxanthin measured for the baby spinach sample by a minimum of one-third compared to only performing one extraction.

Table 5-4 Baby spinach, sample 1E, method variation 5, lutein and zeaxanthin obtained per extraction, multiple extractions

Extraction number	Replicate 1		Replicate 2	
	Percentage of total lutein (%)	Percentage of total zeaxanthin (%)	Percentage of total lutein (%)	Percentage of total zeaxanthin (%)
1	46.7	53.7	56.5	62.7
2	52.1	46.3	42.2	37.3
3	1.2	0	1.3	0
4	0	0	0	0
Combined (µg/100g) *	12,957	383	13,110	416

* Sum of four extractions.

Analysis of two individually analysed hexane extractions was also conducted for Sample F (Table 5-5). The method variations tested with the two individually analysed hexane extractions were 5, 6, 11, and 12. Across these method variations, the first extraction returned between 94.7% and 99% of total lutein measured, and between 95.5 and 100% of total zeaxanthin measured (see Appendix E-5, Figure 9-3 for graphical representation of data). Extractions one and two returned a variable percentage of the total lutein and zeaxanthin with method variation 5 in Samples E and F. In Sample E, the mean total lutein from two extractions was 13,033.5µg/100g and the first extraction contributed to 51.6% of this total. In Sample F, the mean total lutein from two extractions was 7992 µg/100g and the first extraction contributed to 95.4% of this total. Only method variations with two extractions were considered from this stage; and as such, method variations 1–4 were no longer considered.

5.5.1.3 Comparison of method variations 5, 6, 11, and 12

Extraction method variations 5, 6, 11, and 12 were compared for method efficiency in Sample F (Table 5-5). Variations 6, 11, and 12 did not appear to improve lutein and zeaxanthin concentrations compared to variation 5. The recoveries for method variations 5, 6, 11, and 12 were 76%, 72%, 86%, and 71%, respectively. The recovery for method variations 11 was not statistically significantly different to variation 5, and was statistically significantly greater than for variations 6 and 12 ($p = 0.03$, and $p = 0.02$ respectively). As the recovery and measured lutein and zeaxanthin concentrations were not significantly different between variations 5 and 11, variation 5 appeared to be the best method to use as it was more time efficient than variation 11 (no sonication step).5.5.1.4 Comparison of method variations 5, 7, 9, and 10

Table 5-5 Baby spinach Sample 1F, lutein and zeaxanthin obtained per extraction, multiple method variations

Method variation	Extraction number	55. Replicate 1 (% total) †		Replicate 2 (% total) †		Mean of replicates	
		L	Z	L	Z	L	Z
5	1	94.9	100	95.9	100	95.4	100
	2	5.1	0	4.1	0	4.6	0
	Combined (µg/100g) *	8,184	228	7,800	225	7,992	227
6	1	98.1	100	97.9	100	98	100
	2	1.9	0	2.1	0	2	0
	Combined (µg/100g) *	7,427	248	7,177	233	7,302	241
11	1	94.7	95.5	99	99.2	96.9	97.4
	2	5.3	4.5	1	0.8	3.1	2.7
	Combined (µg/100g) *	7,536	243	7,317	247	7,439	245
12	1	95.8	100	95.6	100	95.7	100
	2	4.2	0	4.4	0	4.3	0
	Combined (µg/100g) *	7,236	209	7,365	211	7,300	210

† % total refers to the percentage of total lutein or zeaxanthin measured from extraction one or two.
* Sum of extraction one and two.

Method variation 5 was compared with variations 7, 9, and 10 using Sample 1G (Table 5-6). Variations 9 and 10 returned significantly greater lutein compared to variation 5 ($p = 0.0005$ and $p = 0.0035$ respectively), and variation 7 ($p < 0.0001$, and $p = 0.0002$ respectively). Variation 9 returned significantly less zeaxanthin in Sample 1G compared to variations 5, 7, and 10 ($p < 0.0001$ for all), and no differences were present between variations 5, 7, and 10 (see Appendix E-5, Figure 9-4 for graphical representation of data). The recoveries for method variations 5, 7, 9, and 10 were 77%, 86%, 74%, and 38%, respectively. The recovery for method variation 10 was significantly lower than all other variations ($p = 0.0004$). Measuring lutein in baby spinach was optimal with method variation 9. However, variation 9 was not optimal for measuring zeaxanthin in baby spinach. The optimal method variations for zeaxanthin were variations 5 or 7, as they contained less steps and the percentage recovery were greater than in variation 10.

Table 5-6 Comparison of method variation 5, 7, 9, 10 with Sample 1G

Sample ID	Method variation	Mean ± SD lutein (μg/100g)	Mean ± SD zeaxanthin (μg/100g)
1G (n 7) †	5	9270 ± 448	250 ± 24.4 [a]
	7	9018 ± 316	261 ± 12.1 [a]
	9	10325 ± 464 [b, c]	145 ± 12.1
	10	10149 ± 441 [b, c]	241 ± 13.0 [a]

Abbreviations: ID, identification letter for sample; n, number of replicates analysed per sample; SD, standard deviation. † One-way ANOVA and Tukey's multiple comparison test indicated significant difference between variations for both lutein and zeaxanthin, $p < 0.001$. [a], method variation significantly different to variation 9 for lutein $p<0.0005$; [b], method variation significantly different to variation 5 for lutein $p<0.005$; [c], method variation significantly different to variation 7 for zeaxanthin $p<0.0005$.

5.5.2 Impact of extraction method variations on broccoli, broccolini, baby orange capsicums, and dried goji berries.

Table 5-7 Optimal variation of extraction method for broccoli, broccolini, baby orange capsicums, and dried goji berries

Food	Optimal method variation for lutein	Optimal method variation for zeaxanthin	Method recovery (%)
Broccoli, steamed (n 7)	9	BDL	87%
Broccolini, steamed (n 7)	9	9	88%
Capsicums, orange, baby (n 7)	9	9	83%
Goji berry, dried (n 7)	9	9	73%

Abbreviations: n, number of replicates analysed per sample; BDL, below detection limit

The foods broccoli, broccolini, baby orange capsicums, and dried goji berries also underwent testing to explore differences in recovery using different extraction methods. The optimal method variation for lutein and zeaxanthin was variation 9 for all foods, and the percentage recoveries ranged from 73% to 88% (Table 5-7). Using method variation 9, the mean concentration of lutein in these four foods ranged from 231μg/100g to 2386μg/100g, and 0μg/100g to 2948μg/100g of zeaxanthin (Figure 5-3). Further detail on lutein and zeaxanthin concentrations measured for the different method variations in these four foods is outlined in Appendix E-1 to E-4.

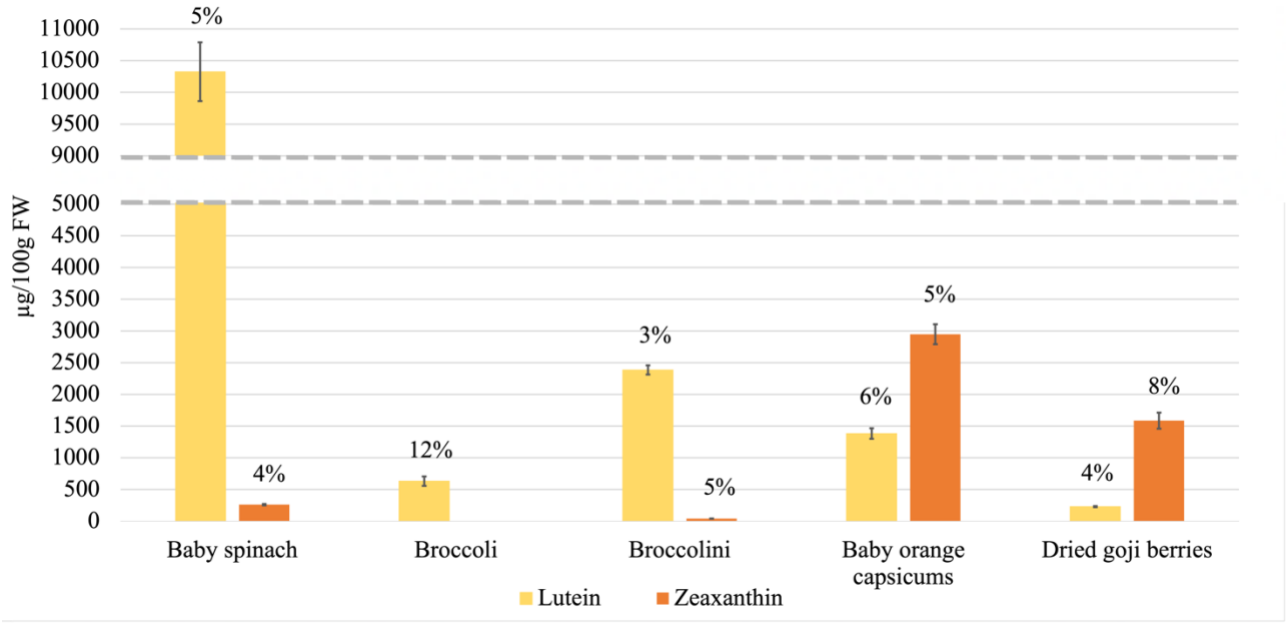

Figure 5-3 Mean concentration of lutein and zeaxanthin with optimal extraction method variation, variation 9, for broccoli, broccolini, baby orange capsicum, dried goji berry.
Error bars indicate standard deviation of the mean. Figure above bar indicates the coefficient of variation as a percentage of the seven replicates analysed. No detectable zeaxanthin was measured in the broccoli sample.

5.6 Discussion

This study investigated optimisation of extraction methods for analysis of lutein and zeaxanthin by HPLC-DAD in five foods for application in developing FCTs in Australia. The five foods tested were baby spinach, broccoli, broccolini, baby orange capsicum and dried goji berry. Method variation 9 was the optimal extraction method for both lutein and zeaxanthin, except for zeaxanthin in baby spinach. Variation 7 would be most appropriate for measuring zeaxanthin in baby spinach due to the greater concentration measured and higher percentage recovery compared to variations 5 or 10. The zeaxanthin concentration of baby spinach measured with variation 9 was approximately 40% lower than with variations 5, 7, and 10. Baby spinach contained low concentrations of zeaxanthin relative to lutein. Thus, in the context of performing large scale analysis of lutein and zeaxanthin for use in developing FCTs, method variation 9 may still be worth consideration for zeaxanthin measurement to reduce analysis costs and optimise measurement of lutein. Variation 9 was effective in this study, however effectiveness may vary with different foods. Changes to steps in the extraction method influenced measurement of lutein and zeaxanthin. Thus, before larger scale analysis, small-scale testing of foods is warranted to ensure the selection of an optimised method variation. Method steps to test include the number of extractions, extraction solvent, saponification steps, and other methods for disrupting cell structures such as chromoplasts to expose lutein and zeaxanthin. A limitation of this study is that the moisture content of individual samples was not measured; therefore any influence of moisture content on lutein and zeaxanthin extraction cannot be

determined. Future studies would benefit from measurement of individual sample moisture content in addition to the extraction steps explored in this study.

5.6.1 Influential extraction steps

5.6.1.1 Multiple extractions

Multiple steps in the method variations influenced the lutein and zeaxanthin concentrations measured. A step that improved assay efficiency was the number of hexane or hexane and dichloromethane (DCM) extractions. Extraction method variations 1 to 4 involved a single hexane extraction. Method variations 5 to 12 involved two hexane or hexane/DCM extractions. A second extraction was impactful when tested on two samples of baby spinach with method variation 5. The lutein in the first of two individually analysed hexane extraction returned 51.1% or 6660µg/100g in Sample 1E, and 95.4% or 7624µg/100g in Sample 1F. The total lutein of all individually analysed extractions combined in Sample 1E was 13,033.5µg/100g, 63% more than the total lutein of 7992 µg/100g found in Sample 1F. A single extraction on both samples would have incorrectly reported a similar total lutein and zeaxanthin concentration. A second extraction appears important for samples with high lutein and zeaxanthin concentrations as the first extraction may reach saturation with carotenoids but not hold all available lutein and zeaxanthin in the sample. Baby spinach is high in lutein and zeaxanthin relative to the three of the four other foods investigated. As two extractions captured >98% of total lutein and zeaxanthin of a high lutein and zeaxanthin containing food like baby spinach, two hexane extractions are required. More than two hexane extractions should be tested in foods with known higher concentrations of lutein and zeaxanthin as seen with baby spinach in this study.

5.6.1.2 Mixed versus single solution extraction solvent.

The second method variation step that improved measured concentrations of lutein and zeaxanthin was the use of n-hexane and DCM in a ratio of 80:20 as the extraction solvent. Use of n-hexane/DCM in a ratio of 80:20 as mixed solvent was reported to result in high recovery rates for zeaxanthin in orange capsicum in a study published partway through completion of this study [263]. This publication was the reason for testing the ratio of 80:20 and method variations 7 to 10 in the present study. The addition of DCM to the n-hexane may have assisted movement of the de-esterified lutein and zeaxanthin into the n-hexane phase after saponification. The use of n-hexane/DCM was only significantly more effective than n-hexane alone when combined with a

saponification step, for example variation 9. This improvement was demonstrated through comparison of variation 9 with variations 5, 7, and 8. Across the different foods, variation 9 returned up to 128% more lutein, and 92% more zeaxanthin than variations 5, 7 and 8. This comparison indicated that n-hexane/DCM was only more effective in combination with a saponification step. Food composition analyses of lutein and zeaxanthin for FCT development must consider both saponification in addition to an appropriate extraction solvent [239].

5.6.1.3 Saponification

Saponification can be an important step for foods that contain the majority of lutein or zeaxanthin in an esterified form, for example orange capsicum [263]. Saponification can also contribute to carotenoid loss and reduction in carotenoid stability. Carotenoids in solution may be sensitive to light, heat, acid or oxygen exposure. Reducing the method time and exposure to these factors is important to reduce carotenoid loss. A saponification step has shown mixed results in recovery of lutein across different foods [270, 271]. The addition of a saponification step of 150µL of 10 molar KOH and incubation in a light protected water bath at 45 °C for 30min was beneficial to lutein and zeaxanthin recovery for all foods except zeaxanthin in baby spinach. The greater concentrations of up to 128% for L and 92% for zeaxanthin measured with variation 9 compared to variations 5 and 7 isolate the saponification step as being influential in the improved assay return.

The use of MeOH NaOH in place of KOH as the saponification solution appeared to further free esterified lutein and zeaxanthin for analysis. Variation 10 reported similar total lutein and zeaxanthin concentrations when compared to variations 5, 7, and 9. However, the recovery measured by spiked lutein samples with use of the MeOH NaOH step was lower than the other variations for four of the foods: baby spinach 38%, broccoli 60%, broccolini 55%, and dried goji berry 33%. These lower recovery rates may not only indicate release of esterified lutein and zeaxanthin but also loss of free lutein and zeaxanthin in variation 10. This release and loss suggest the data issued from variation 10 may be unreliable. Additionally, the potential release and loss may explain how the lutein concentration in baby spinach measured in variation 10 remained higher than with variations 5 and 7 despite a low method recovery. This occurrence highlights the importance of testing multiple method variations. The use of lutein spiked samples alone was not adequate to determine if an extraction method was capturing all lutein and zeaxanthin present as it did not provide an indication of whether esterified lutein and zeaxanthin was being captured. Testing multiple extraction methods is needed to optimise the freeing of esterified lutein and zeaxanthin whilst minimising lutein and zeaxanthin loss.

5.6.1.4 Sonication

Sonication was tested as a method to further disrupt cell membranes and expose lutein and zeaxanthin from structures such as chloroplasts or chromoplasts. In broccoli, sonication may have contributed to improved return of lutein. The sonication step in combination with saponification (variation 4) improved return of lutein for broccoli compared to variation 3 but was no different to variations 1 or 2 (see Appendix E-1, Table 9-6). Sonication may contribute to improved recovery for some foods; however, due to time and financial restraints it was not tested whether sonication would improve variation 9. Two mechanical disruption steps of blending were already present and other steps (i.e. number of extractions, extraction solvent, and saponification) were prioritised due to their potential for greater influence. Future studies may benefit from testing the impact of sonication on recovery when testing for the optimal extraction method.

5.6.2 Measured lutein and zeaxanthin concentrations in comparison to pre-existing literature and databases

The lutein and zeaxanthin values measured for the five foods in this study justify the need for local Australian lutein and zeaxanthin FCTs. The lutein and zeaxanthin concentration of the five foods were not consistently aligned with pre-existing literature and databases [138, 229]. The 'true' values of reported concentrations of lutein and zeaxanthin in these five foods may be higher than reported in some cases as they were not always measured with variation 9. Only one sample of steamed broccoli had detectable zeaxanthin of 33µg/100g and was measured with variation 2. The mean lutein concentration of the nine broccoli samples was 841µg/100g (range: 276–1,150µg/100g), with only one sample reporting a value below the FSANZ reported mean value of 352.5µg/100g lutein (range: 0.5–800µg/100g) [272]. The USDA tables report a mean lutein and zeaxanthin value of 1,080 µg/100g (range: 447–1,940µg/100g) for boiled and drained broccoli [138]. In the context of estimating Australian dietary lutein and zeaxanthin intake, the use of the FSANZ value could underestimate intake by 58% and USDA overestimate by 28% per 100g of broccoli. The variability in lutein and zeaxanthin values highlight the importance of representative lutein and zeaxanthin values in FCTs to reduce error when monitoring dietary lutein and zeaxanthin intake.

The mean lutein steamed broccolini concentration was 2,540µg/100g (range: 2,114–3,121µg/100g), 79% above the FSANZ reported value for boiled and drained broccolini of 1,417µg/100g (zeaxanthin not reported) [229]. Broccolini is not reported in the USDA tables [138]. Therefore, dietary lutein and zeaxanthin intake from broccolini would be underestimated with the use of the FSANZ or USDA FCTs.

The lutein and zeaxanthin concentration of the four samples of baby orange capsicum were similar to concentrations in some cultivars of orange capsicum that have been reported in the literature [263]. In this study, the mean concentration was 523µg/100g (range: 170–1,384µg/100g) for lutein and 697µg/100g (range: 167–2,948 µg/100g) for zeaxanthin. An Australian study of seven orange appearing capsicum varieties measured mean ± SD zeaxanthin concentrations between 1.9±0.1mg/100g and 28±8.5mg/100g [263]. The zeaxanthin values measured in this study were baby capsicums rather than mature capsicums. Maturity of a fruit or vegetable is known to impact carotenoid concentrations [170, 237]. The concentrations of zeaxanthin in baby orange capsicums in this study aligns with lower zeaxanthin concentration varieties previously reported for mature orange capsicums [263]. The USDA and FSANZ tables do not report values for orange capsicum or baby orange capsicum [138, 229]. The USDA tables report a lutein and zeaxanthin value for raw green capsicum of 341µg/100g which may underestimate lutein and zeaxanthin intake from baby orange capsicums in Australia by 72%.

The mean baby spinach values were 8,905µg/100g (range: 6,842–13,034 µg/100g) for lutein and 284µg/100g (range: 227–400 µg/100g) for zeaxanthin. All seven samples reported at least a 17% greater lutein and 19% greater zeaxanthin concentration than the mean values reported by the USDA tables. The mean USDA lutein concentration was 5,830µg/100g (range: 5,320–7,110µg/100g), and zeaxanthin concentration was 191µg/100g (range: 0–511µg/100g) [138]. The USDA baby spinach lutein and zeaxanthin values were measured as part of a larger analysis capturing more carotenoids than just lutein and zeaxanthin [138, 273]. Baby spinach lutein or zeaxanthin is not reported by FSANZ currently [229]. Estimation of lutein and zeaxanthin from Australian baby spinach intake using the USDA tables may underestimate intake by 34%. The differences in food lutein and zeaxanthin concentrations observed in this study compared to both the USDA and FSANZ FCTs highlight the potential impact possible from non-representative FCTs on investigations of the relationships between dietary intake and disease risk and management [4, 235]. The observed differences also support the pursuit of a targeted program to develop Australian lutein and zeaxanthin FCTs.

5.7 Conclusion

The differences between lutein and zeaxanthin values measured in this study and those reported from the FSANZ and USDA FCTs justify the need for a larger lutein and zeaxanthin Australian dataset. The USDA FCTs for lutein and zeaxanthin are large and thus are often used to calculate dietary lutein and zeaxanthin intake [67]. Translated into dietary lutein and zeaxanthin intake, these differences values could have significant impact in over or underestimation of dietary lutein and zeaxanthin intake. The over or underestimation of dietary lutein and zeaxanthin intake translates into in

accurately assessing diets for the purpose of disease risk and management. The analysis methods used in FCTs are an important consideration when interpreting past and future research investigating the relationship between dietary intake and disease risk and management. Specific to the investigation of dietary lutein and zeaxanthin and age-related macular degeneration, comprehensive Australian FCTs for lutein and zeaxanthin are needed.

5.8 Summary

Chapter 5 successfully addressed thesis objective 4, the investigation of an appropriate method for the analysis of food L and Z concentrations suitable for building local Australian FCTs. High biological variability was present in samples of Australian foods. Methodological optimisation is needed for each food to best capture L/Z concentrations for FCTs. The findings of Chapter 5 relate to criteria 2 and 3 of the research framework (Figure 5-4). The findings indicate criterion two is able to be met outside the US context. However, the existing data available to meet criterion three may not have been optimised for L/Z analysis and is minimal outside of the US, such as in Australia. The findings relating to criteria 2 and 3 subsequently impact estimations of dietary L/Z intake measured in studies relating to criteria 4 to 8 (Figure 1-1, page 31).

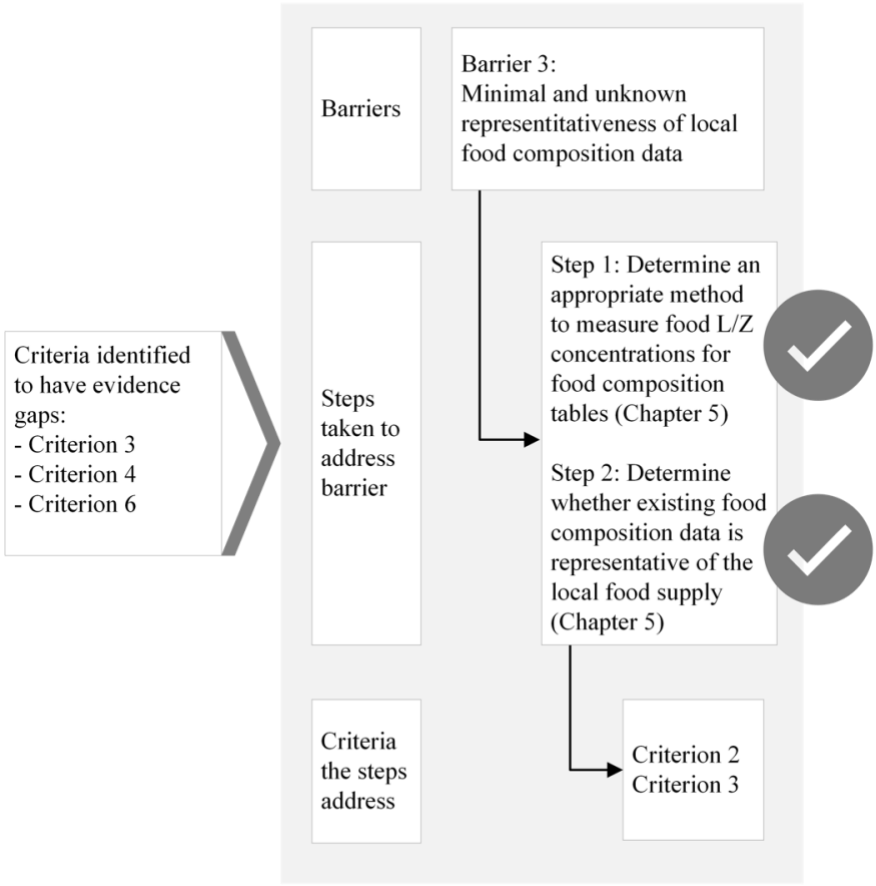

Figure 5-4 Steps addressed as part of Chapter 5 to improve the lutein and zeaxanthin evidence base related to the 9-criteria by Lupton et al. [2]

Chapter 6 Discussion

This chapter provides a cohesive discussion about work completed in this thesis, integrating the adapted research framework demonstrated in Figure 6-1. [1] It discusses the purpose of the thesis and summarises key outcomes from each chapter. It also expands on the contribution of this research, with its strengths, limitations, and implications for dietary intake recommendations.

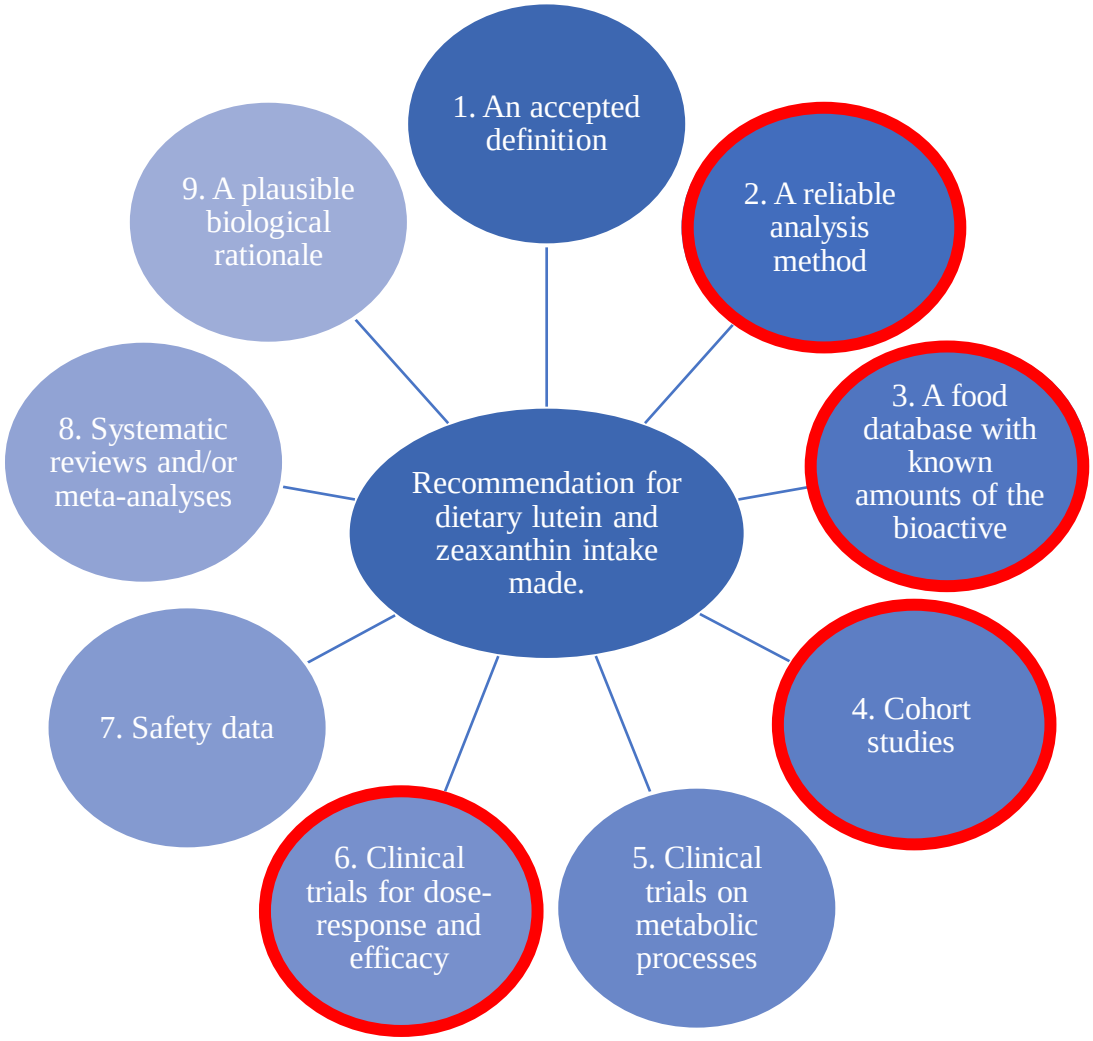

Figure 6-1 Criteria identified to have evidence gap, adapted from Lupton et al.[2]

6.1 Summary of thesis

The rationale for this work arose from the limitations identified with criterion 6 in the proposal by Ranard et al.[1] for L/Z to be considered for a dietary target recommendation. It was identified in the narrative review that there was minimal clarity surrounding the dose-response relationship in humans between dietary L/Z intake and MPOD as a surrogate indicator of macular health. Thus, the current evidence base was deemed insufficient to meet criterion 6 of the research framework

(Figure 6-1, page 161). A need for valid and quantitative measurement of habitual dietary L/Z intake was identified as a key barrier to meeting criterion 6.

The overarching research question of this work was therefore: How can habitual dietary L and Z intake be validly and quantitatively estimated to investigate links to ocular health?

To answer this research question, the aims of this thesis were to:

1. Develop and validate a method for quantitatively capturing habitual dietary L/Z intake.
2. Develop and validate a method to investigate whether blue light exposure from usual electronic device use impacts macular L/Z concentrations.
3. Identify an appropriate method to analyse L/Z concentrations in local foods to increase data available in the Australian food composition tables (FCT).

A significant barrier to determining how dietary L/Z intake can be validly monitored was that no appropriate dietary intake tool was available to quantitatively measure dietary L/Z (section 1.3). An additional two barriers were identified in the literature reviewed throughout this thesis (section 3.1 and 5.1). These two barriers were the potential impact of ED blue light (BL) exposure on MPOD, and the paucity of L/Z data listed in local Australian FCTs. These three barriers provided the justification for the four thesis research objectives:

1. Development and validation of two dietary screeners designed to capture habitual dietary L/Z intake over one week and one month respectively in Australian and UK adults.
2. Development and validation of a questionnaire to capture usual ED use behaviours in Australian and UK adults.
3. To investigate the associations between ED use, dietary L/Z intake and MPOD in healthy Australian adults, using the newly developed tools.
4. Investigation of an appropriate extraction method for analysing food L and Z concentrations suitable for building local Australian FCT.

These objectives aimed to address the three identified barriers to validly measuring habitual dietary L/Z intake, a key factor when determining diet-disease relationships, through four original studies (Figure 6-2). These four studies were developed using the 9-point criteria developed by Lupton et al.[2] as a research framework. The use of these nine criteria as a framework ensured study outcomes were relevant to improving the quality of the body of L/Z research to meet the nine criteria. The impact of the study outcomes on these three identified barriers are outline in Figure 6-2 and throughout the discussion.

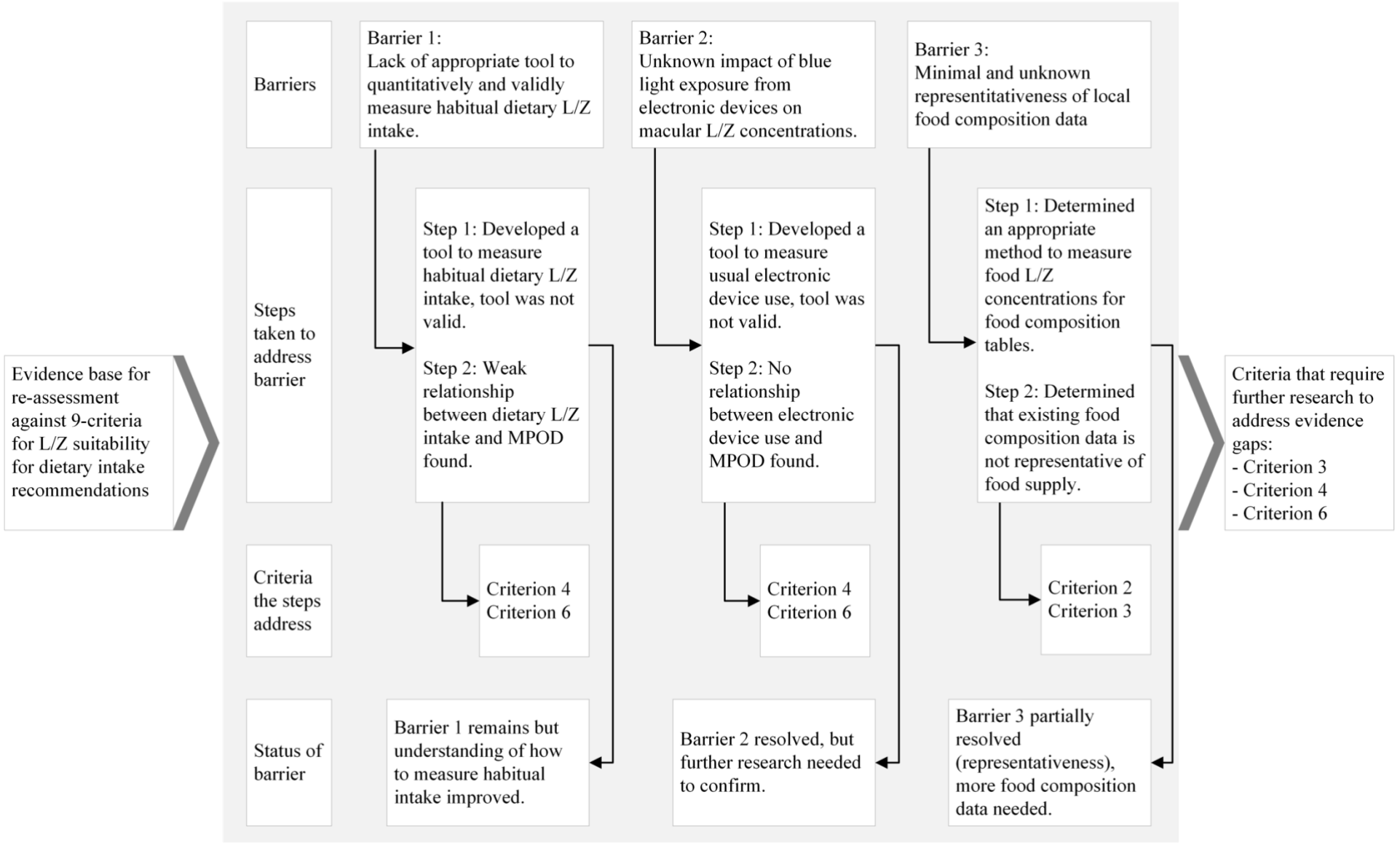

Figure 6-2 Remaining barriers to lutein and zeaxanthin meeting the 9-criteria by Lupton et al. [2] to support a dietary intake recommendation

Findings from the original studies are discussed in relation to the three identified barriers to answer the overall thesis research question: How can habitual dietary L and Z intake be validly and quantitatively estimated to investigate links to ocular health?

6.2 Barrier 1: appropriate tool to monitor dietary L/Z intake

The paucity of specific, quantitative, and validated tools to monitor habitual dietary L/Z intake was identified in the narrative review (section 1.3). This barrier formed the basis for the aims of this thesis (Figure 6-2, page 163). To address this barrier and meet the aims of the thesis a dietary screener was the tool developed to explore quantitative measurement of habitual dietary L/Z intake (Chapter 2 and Chapter 4). The outcomes of this thesis demonstrated that habitual dietary L/Z intake cannot yet be quantitatively estimated validly, however steps to continue to improve the validity of dietary intake tools have been identified.

6.2.1 Summary of Chapter 2 and Chapter 4 studies

The objective to develop the monthly screener (MS) and weekly screener (WS) was achieved with the screeners containing 91 food items; 25 fruits, 39 vegetables, six grains, 12 meat and meat alternatives, three dairy and alternatives, and six discretionary foods. However, the MS and WS were not valid due to demonstrated poor Bland-Altman plot agreement, and the objective to validate the screeners was not met (Chapter 2).

The cross-sectional study (Chapter 4) investigated associations between ED use, dietary L/Z intake, plasma L/Z, and MPOD in healthy Australian adults. The multiple linear regression model to predict MPOD from ED use, dietary L/Z intake, sex, and age was not statistically significant. MPOD was significantly correlated with plasma L/Z ($r = 0.32$, $R^2 = 0.09$, $p = 0.002$), and plasma L/Z was significantly correlated with dietary L/Z intake from the MS ($r = 0.28$, $R^2 = 0.35$ $p = 0.008$). The multiple linear regression to predict plasma L/Z from dietary L/Z intake, body fat percentage, sex and age was statistically significant, $F(4, 81) = 23.16$, $p = <0.001$, adjusted $R^2 = 0.51$.

The screener development and cross-sectional study median (25^{th} to 75^{th} percentile) daily intakes from the MS were 3.1 (2.2 – 4.5) mg/day and 4.6 (2.7 – 7.4) mg/day respectively. The contribution of food groups and foods to total L/Z intake from the MS were similar between the Australian participants in the studies. In both studies 91% of intake came from vegetables. The top contributing food was baby spinach in both studies and the remaining five foods were pumpkin, broccoli, raw orange carrot, zucchini and lettuce just varying in order of contribution between the studies. It is important to note that 29 of the 96 participants that enrolled in the cross-sectional study also enrolled in the screener development study.

6.2.2 Accurate capture of dietary lutein and zeaxanthin intake

The poor validity of the monthly L/Z screener demonstrated in Chapter 2 may partially explain the weak correlation between dietary L/Z intake and plasma L/Z, and non-existent correlation between dietary L/Z intake and MPOD found in Chapter 4. The outcomes of the cross-sectional study align with the proposed understanding that MPOD levels are influenced by circulating plasma L/Z, and plasma L/Z is influenced by dietary L/Z intake. [232] Thus, as dietary L/Z intake was demonstrated to be difficult to accurately capture in the screener validation study, a weak correlation to plasma L/Z and no correlation to MPOD could be expected. This outcome may help to make sense of findings from prior research. Previous research about the correlation between dietary L/Z intake, plasma L/Z intake and MPOD has found mixed results, from no association up to strong associations. [55, 63, 107, 111, 130, 232] The thesis findings suggest heterogeneity in reported outcomes are partially explained by the use of dietary intake tools with poor validity, and sampling of blood at times that may align poorly with the dietary tool recall timeframe. It is acknowledged that the heterogeneity in results can be explained by established confounding factors such as inter-individual genetic differences resulting in difference in dietary L/Z bioavailability, and L/Z accumulation in tissues. [56, 111] However, the role of using a valid dietary intake tool, and appropriately timed blood sampling are key findings to determine the design of future research investigating the relationship between dietary L/Z intake, plasma L/Z, and MPOD.

Developing a valid tool to quantitatively capture habitual dietary L/Z intake was an aim of this thesis that was not able to be met. The poor validity outcomes in the screener development study, whilst valuable, aligned with the majority of prior research that had attempted to validate L/Z intake with non-L/Z specific FFQs. [143, 151-153] A comparison of the thesis results with findings from earlier studies indicates that questionnaire validity overestimation may be high when statistical analysis is solely reliant upon correlational statistics. Daily dietary L/Z intake from the MS and CWS were both significantly correlated with the 24DRs, and more strongly so than found many prior studies (Table 6-1). [152, 153] However, comparisons of the correlation outcomes in contrast to the Bland-Altman plot analysis found in the screener development study demonstrate linear correlation statistics to be a potentially misleading indicator of tool validity.

Table 6-1 Daily dietary lutein and zeaxanthin intake correlations between dietary intake tools

	Population	Tool Comparison	Correlation Coefficient	Deattenuated correlation coefficient	p value
Thesis screener development study (Chapter 4)	Australian Cohort (n 31)	MS2 and 24DR (n 31)	0.58	0.35	<0.001
		CWS [4] and 24DR [8]	0.70	0.67	<0.001
	UK Cohort (n 23)	CWS [3+] and 24DR [6+]	0.62	0.12	0.002
	Combined Cohort (n 35)	CWS [4] and 24DR [8]	0.75	0.57	<0.001
McNaughton et al. (2005) [152]	Australian (n 28)	FFQ with 6-month recall timeframe and 12 food records	0.40	0.19 [a]	<0.05
Satia et al. (2009) [153]	African American (n 28)	FFQ recall timeframe 1 month and 4 24DR	0.51 [b]	-	≤0.0001
	White American (n 81)	FFQ recall timeframe 1 month and 4 24DR	0.49 [b]	-	≤0.0001
Cena, Roggi, & Turconi (2008) [91]	Italian adults (n 87)	30-item FFQ with 1-month recall	0.94 [c]	-	<0.001
		timeframe, and 7-day diet record, and blood L/Z	0.76 [d]		<0.001

[a] Validity coefficient calculated by the method of triads with two tools and plasma L/Z. [b] Adjusted correlation coefficient reported, adjusted for age, sex, education, body mass index. [c] Correlation coefficient between FFQ and food record. [d] Correlation coefficient between FFQ and blood L/Z. Abbreviations: UK, United Kingdom; MS2, monthly screener 2; 24DR, 24-hour diet recall; CWS, combined weekly screeners; FFQ, food frequency questionnaire; [4], mean intake per day from the four weekly screeners; [8] mean intake per day from the eight 24-hour diet recalls; [3+], mean intake per day from 3 or more weekly screeners; [6+] mean intake per day from 6 or more 24-hour diet recalls; n, number of participants.

The outcomes of the screener development study indicate that evaluating the validity of the instrument by Bland-Altman plot is more rigorous than correlational statistics alone. The reporting on the contribution of each food to total L/Z intake also adds rigour to the analysis. The poor agreement between the screeners and 24DRs was found to be related to a mixture of misestimation of intake and missed capture of intake (section 2.6). The cross-sectional study outcomes exemplified the impact of missed capture of intake (Chapter 4). The cross-sectional study combined the use of a single 24DR, the MS, plasma L/Z, and MPOD. The outcomes provided new insight into the capture of dietary L/Z intake. The issue and impact of missed capture was demonstrated with

L/Z intake from the single 24DR. The intake from the 24DR showed no individual correlation to either plasma L/Z or MPOD. The lack of relationship supports the use of a longer dietary recall timeframe, such as a month as intake from the prior 24-hours was not reflected in the blood L/Z measure taken the same day as the 24DR. The lack of relationship also aligns with the half-life of L and Z in the blood that has been variably reported, with studies indicating it to be between 5 and 76 days. [120-122] It is therefore unlikely that a single day of dietary intake would reflect plasma concentrations. Similarly, whilst responsive to supplementation, MPOD is reported to be stable with a steady lifestyle (such as dietary intake, weight stability) and health status. [46] The lack of individual association between the 24DR, blood L/Z, and MPOD from the cross-sectional study is an important outcome when considered together with screener development study outcomes. It is important in the context of understanding what timeframe, or how many days of dietary intake may be needed to be reflective of habitual L/Z intake. In the screener development study participants demonstrated high inter-day variability in L/Z intake. Eight 24DRs over four weeks, representative of 29% of four weeks' worth of intake, was selected as it was hypothesised to be an adequate number of days to capture inter-day variability in L/Z intake. In a validation study by Yuan et al. [143] 3 24DRs over 12 months returned poor correlation with plasma L/Z ($r < 0.45$). Therefore, it was proposed in this thesis more than four days was needed, thus eight was selected. However, the thesis outcomes suggest that capturing 8 of 28 days is still not enough to be representative of habitual intake due to high inter-day variability in participant intake. This high inter-day variability is likely related to the non-ubiquitous L/Z distribution across foods, and contributed to the poor agreement between the 24DRs, CWS and MS.

6.2.2.1 Variability in dietary lutein and zeaxanthin intake and measurement method choice

This finding regarding high variability in intake is important for two reasons. The first reason is the verification that a dietary intake method such as a valid screener or FFQ, rather than acute recall method like 24DR, may be necessary to adequately capture habitual L/Z intake that it is reflected in measures of blood L/Z and MPOD. A dietary intake tool that reflects blood L/Z or MPOD is an important step in enhancing the evidence base for criterion 6 (Figure 6-1, page 161). The outcomes of the screener development and cross-sectional studies cohesively support continued efforts to improve the validity of these screeners or a similar tool.

The recall timeframe of a month for the MS was informed by the reported half-lives of L and Z with the hypothesis that reported intake would align with blood L/Z concentrations. Similarly, the WS was developed with the intention that it would not be used as a once off measure but repeated over the timeframe of interest. The screener validation study indicated that both the WS and MS require refinement to reduce misestimation of reported intake and improve their accuracy. The cross-

sectional study demonstrated that despite the low MS validity, a recall timeframe of four weeks may be adequate to reflect plasma L/Z concentrations. This was demonstrated by the significant correlation between dietary L/Z intake from the MS and plasma L/Z concentration ($r = 0.28$, $R^2 = 0.35$, $p = 0.008$). The L/Z screeners require further work to improve their validity in capturing L/Z, however, they may have an appropriate recall timeframe to reflect plasma L/Z concentrations in healthy adults.

The month timeframe is also supported by the short FFQ developed and validated in 87 Italian females aged 20-25 years. [91] This 30-item fruit and vegetable FFQ with a recall timeframe of a month was significantly correlated with a 7-day diet record and plasma L/Z measure (Table 6-1). The FFQ completed was dietitian-administered by interview, and both the FFQ and 7-day diet record completed with the assistance of a photographic atlas. L/Z intake from the tools was determined using the USDA database. The FFQ and 7-day record showed close agreement via Bland-Altman plot analysis with a mean difference (FFQ minus records) of -24.5 μg/day with 95% LOA from -50.6 μg/day to 99.6 μg/day. [91] The strengths and limitations of this study have been previously discussed (section 2.1.2). There are multiple potential reasons the Italian FFQ may have performed better than the L/Z screener developed in the current thesis. One is that the FFQ was completed with the assistance of a dietitian and photographic atlas which likely enhanced the accuracy of the report, as specific prompts trigger memory and portion size estimation. [91] Another is the difference in the number of questionnaire items, and comparative method selected to validate the FFQ. It is also possible that there is less inter-day variability in dietary intake within this population. However, as detail on which foods contributed most to the total intake was not reported, the inter-day variability cannot be clarified further. As the Italian FFQ is only 30 items long (20 vegetables and 10 fruits) it is unlikely to be representative of habitual intake. However, with the exception of egg, the list does contain the top six contributing foods found in this thesis for the Australian and UK populations. A key benefit of a FFQ or diet screener is its ability to be economically disseminated to large numbers of participants who can complete the tool independently. Administering the L/Z screeners via interview with a dietitian would significantly reduce the feasibility. However, the outcomes of the Italian FFQ study support the recommendation of adding the use a photographic atlas to improve the validity of the data captured by the L/Z screeners developed in this thesis (section 2.6). [91]

This Italian FFQ study findings also support this thesis' outcome that L/Z dietary intake validation studies solely reliant on correlational statistics should be interpreted with caution. In this thesis, the poor agreement measured by Bland-Altman plot co-occurred with a moderately strong correlation ($r = 0.70$, $p<0.001$) between the CWS and 24DR. In the Italian FFQ study, good agreement measured by Bland-Altman plot co-occurred with a strong correlation between FFQ and 7-day diet record ($r =$

0.94, p<0.001). [91] In both studies the Bland-Altman plot provided more valuable information regarding any random or systematic bias that may be present compared to linear correlational statistics. [164] Together, the results of these two studies suggest that in the setting of dietary L/Z tool validations, a strong correlation coefficient above 0.90 between two tools may be needed to indicate a valid tool. In relation to prior research, tools deemed valid with correlation coefficients lower than that observed in this thesis and the study by Cena, Roggi, & Turconi [91] may need reconsideration. In future research, to confirm whether linear correlational statistics are representative of an L/Z intake tool validity, the combined use of correlational statistics and a Bland-Altman plot analysis is warranted.

In the cross-sectional study, plasma L/Z concentrations were associated with MPOD status and investigating the capability of the screener to reflect plasma L/Z concentration is a step in understanding how dietary L/Z intake relates to MPOD status. In this study, the multiple linear regression to predict plasma L/Z from dietary L/Z intake from the MS, body fat percentage, sex and age was statistically significant, $F(4, 81) = 23.16$, $p = <0.001$, adjusted $R^2 = 0.51$. The beta standardised coefficient for the MS was 0.493 and was significantly correlated with plasma L/Z, p<0.001. Age, body fat percentage and sex were also significant predictors of variance in plasma L/Z concentrations. An objective measure such as blood L/Z is important in understanding the relationship between dietary L/Z intake and MPOD. As previously suggested (section 2.1.4), the outcomes of the cross-sectional study support that plasma L/Z cannot be a complete substitute for measuring dietary intake as other factors such as body fat percentage appear to contribute to variance in plasma L/Z levels. [130] Additionally, a focus only on plasma L/Z will not allow for criterion 6 of the research framework, and subsequent target intake recommendation to be met for L/Z. Improvement to the accuracy of reporting by participants with the screeners may improve its association to plasma L/Z and strengthen the understanding of how dietary L/Z intake relates to MPOD (relates to criteria 4 and 6).

6.2.2.2 Variability in dietary lutein and zeaxanthin intake in relation to prior research

The second reason the finding of high variability in dietary L/Z intake is an important contribution of this thesis is its application to the interpretation of prior research. In particular, prior research that has relied upon prospective dietary intake methods over a small number of days as the study method, or FFQ validation method. The thesis findings of poor L/Z screener validity and lack of relationship between dietary L/Z and MPOD observed raise questions of the interpretations of previous research investigating dietary L/Z and MPOD, or risk of AMD.

Many large cohort studies have investigated dietary L/Z intake and AMD risk with inconsistent results. [274] Part of this inconsistency may be explained by the lack of available dietary intake methods specifically validated to capture habitual dietary L/Z. The Australian Blue Mountains Eye Study is one such cohort study that exemplifies potential issues with the validity of dietary L/Z intake collected. Study outcomes from a 10-year follow up of 2454 adults 45 to 93 years indicated that those with above median intakes of L/Z had a reduced risk of developing soft or reticular drusen (a surrogate risk marker of AMD development), relative risk 0.66 and 95% CI 0.48 to 0.92. Additionally, those in the top tertile of L/Z intake had a reduced risk incidence of neovascular AMD, relative risk 0.35 and 95% CI 0.13 to 0.92. Mean ± SD L/Z intake per day was 0.826 ± 0.482 mg and the top tertile was ≥0.942 mg. The dietary intake method used in this study relied upon the USDA FCTs and was a semi-quantitative FFQ 145 items long with a 12-month recall timeframe. [13] The FFQ was validated in a subset of 79 participants against three weighed food records completed 4 months apart. However, L/Z were not included in the analysis. [92] Beta-carotene was assessed, and the adjusted Pearson product moment correlation was 0.49 and classification into the correct quintile was 35%. As identified in the thesis narrative review, and screener development study, a tool specific to L/Z or at least specifically validated to assess L/Z is needed. Arguably, even if L/Z intake was analysed, the outcomes of the screener development study suggest a total of 12 days of intake captured over a year would not be representative of habitual intake, therefore over- or underinflating FFQ validity. In this thesis it was observed that habitual dietary L/Z was highly variable within Australian and UK participants and intake was dominantly reliant on moderate-high concentration vegetables such as baby spinach and broccoli. The missed capture of habitual dietary L/Z intake could incorrectly strengthen or weaken results. With the plausible biological mechanism that exists, it is possible that the relationship between dietary L/Z and risk of AMD was stronger than observed in the Blue Mountains Eye Study. However, it remains unknown as the FFQ used was not validated to capture habitual dietary L/Z. The impact of dietary L/Z intake in prior cohort studies must be interpreted with caution. The evidence base does not yet demonstrate a clear dose-repose relationship between dietary L/Z intake and MPOD or AMD risk. These findings suggest criteria 4 and 6 are not met (Figure 6-2, page 163). Improving the validity of methods to capture habitual dietary L/Z intake is of high importance.

In relation to dietary L/Z intake, a key finding of the thesis is that prior research investigating dietary L/Z should be interpreted with caution. Many studies have not included L/Z in validation of the tools used, or have attempted to validate dietary intake questionnaires measuring L/Z with correlational statistics. The outcomes of this thesis indicate this may be highly inappropriate and overestimate the validity of the questionnaire. Further research to improve dietary measurement

methods able to capture L/Z intake is justified. The outcomes of the screener development and cross-sectional studies relate to criteria 4 and 6 of the thesis research framework (Figure 6-2, page 163). Understanding the poor validity of how habitual dietary L/Z has been captured in prior research makes the interpretation of prior research outcomes more unclear. Outcomes are likely stronger than previously reported, however they could also be weaker. The inability to accurately interpret the outcomes of prior research highlighted by this thesis indicate that the evidence base to support criteria 4 and 6 is not yet strong enough. Making steps toward understanding how to adequately capture habitual L/Z intake will strengthen future cohort and dose-response investigations thus moving L/Z closer to meeting the nine criteria.

6.3 Barrier 2: Unknown impact of blue light exposure from electronic devices on macular lutein and zeaxanthin concentrations

Blue light exposure from ED was hypothesised to negatively impact MPOD status. Therefore, ED use was deemed a potential confounding factor when attempting to determine the relationship between dietary L/Z intake and MPOD status. The chronic and frequent use of EDs is a relatively new and potentially impactful environmental exposure to BL. Exposure to ED BL and its impacts on MPOD was therefore investigated as part of the EDUQ development study (Chapter 3) and cross-sectional study (Chapter 4).

As a result of L/Z acting through direct antioxidant activity in response to photochemical damage from ED BL exposure, there may be increased turnover of L/Z in the macula, therefore impacting MPOD status. [10, 172] This increased turnover of L/Z at the macula may then influence how blood L/Z concentrations, and dietary L/Z intake are related to MPOD status. Additionally, increased macular L/Z turnover has potential to influence the target dietary L/Z intake determined necessary to maintain a protective MPOD status. Therefore, it was important to understand whether BL from EDs is impacting MPOD.

Chapter 3 addressed thesis objective 3 and was the development and validity evaluation of the EDUQ, a novel questionnaire to capture ED use behaviours. The EDUQ and 24-hour device use diary (24DUD) are new contributions to this research field. Part of the second thesis objective relating to developing the EDUQ was met (See Appendix C-1 for the EDUQ). The validation component of the first thesis objective was not met as the EDUQ demonstrated poor validity. The inability for participants to consistently recall hours of daily ED use indicates the presence of memory recall bias which has been reported in studies attempting to capture similar behaviours. [204]

Although the EDUQ demonstrated poor validity, this was the first study to capture detailed behaviours of usual ED use (not just handheld devices). The prospective 24DUD captured intra- and inter-day patterns in ED use that provide novel insight into how EDs are being used in this population. In a healthy, predominantly female, and tertiary educated population, the use of ED is a large component of most individual's days. In relation to the proposed mechanism for macular damage from BL exposure due to ED use, an important aspect of ED use behaviour has been observed in this thesis. The proposed mechanism is photochemical damage. The two factors that influence the likelihood or severity of photochemical damage are the duration and wavelength of light exposure. [172, 180] The thesis study provided in depth insight into the duration of ED BL exposure over a day. Using the 24DUD it was observed that both cohorts of participants used ED continuously for hours at a time (Table 6-2). Further to this, participants consistently indicated that their ED use is stable or increasing. Compared to 1 year ago 63% of Australian and 42% of UK participants indicated no change in their use of EDs, while 32% of Australian and 50% of UK participant indicated an increase in ED use. In contrast, compared to 5 years ago, 72% of Australian and 88% of UK participants indicated an increased in their ED use (Appendix C-4, Table 9-3). The duration of BL exposure from EDs is long, repeated, and increasing for many participants indicating high potential for negative impacts at the macula. Thus, despite the low validity of the EDUQ, the cross-sectional study was an important first step in understanding whether ED exposure is currently reflected in MPOD status.

Table 6-2 Example of participant electronic device use patterns over a day

Example Participant	Time of day	Hours	Device being used
Aus1	7:45am – 12:15pm	4.15	Computer
	1:00pm – 2:45pm	1.50	Computer
Aus2	7:00am – 3:00pm	8.52	Computer and 1.35 hours of handheld spread throughout
Aus3	7:30am – 6:15pm	9.08	Computer and 0.92 hours of handheld used intermittently throughout
Aus4	5:45am – 7:00am	1.17	Handheld
	7:45am – 12:00pm	3.83	Computer and 0.25 hours of handheld
	1:15pm – 6:00pm	4.75	Computer and 0.5 hours of handheld
	6:30pm – 9:00pm	2.50	Handheld
UK1	7:15pm – 10:45pm	3.5	Television
UK2	9:45am – 12:00pm	2.33	Computer
	1:00pm – 2:30pm	1.40	Computer
	4:00pm – 5:15pm	1.22	Computer
	6:00pm – 11:45pm	3.73	Computer
UK3	12:00am – 4:15am	4.42	Handheld
UK4	9:30am – 12:30pm	2.00	Television
	1:45pm – 6:00pm	4.24	Television

Example participant column: participant country of represented by Aus or UK, and differing number indicates a different participant. Abbreviations: Aus, Australia; UK, United Kingdom

The cross-sectional study achieved thesis objective 3 and was the first study to the authors' knowledge to investigate associations to MPOD with ED use dietary L/Z intake via multiple linear regression analysis. This study found that ED use and MPOD were not correlated and the multiple linear regression to predict MPOD from ED use, dietary L/Z intake, sex, and age was not statistically significant, $F(4, 87) = 1.396$, $p = 0.24$, adjusted $R^2 = 0.06$. The results of this study suggest that when using MPOD as an indicator of macular health, chronic ED use does not appear to negatively impact macular health. The absence of a relationship between MPOD and ED use also indicates that ED use is not currently a significant confounding variable when investigating the relationship between dietary L/Z and MPOD. As previously outlined in (section 5.5), the lack of relationship found between ED use and MPOD does not necessarily rule out that a relationship is present.

A relationship may not have been found due to poor validity of the EDUQ, the HFP method used to measure MPOD, selection of MPOD as the indicator macular BL impact, study sample size, and participant characteristic homogeneity. Whilst a more valid EDUQ may not have changed the outcomes of the cross-sectional study, it cannot be ruled out that negative implications of chronic BL exposure from EDs exist. When trends of increasing habitual ED use are taken into consideration, the possibility that ED BL exposure could impact MPOD (or other markers of macular health) remains plausible. In the thesis studies, a large percentage of participants reported increases in ED use in the last one or five years. In the cross-sectional study, compared to 1 year ago 34% of participants indicated ED use had increased, while 54% reported no change. In contrast, compared to 5 years ago 80% participants indicated ED use had increased, while 12% no change. It should be noted that 34 of the 96 participants enrolled in the cross-sectional study also participated in the EDUQ development study. Thus, the participant crossover partially contributed to the similar trends in ED use change in the last one and five years. Regardless of the crossover, the trends in ED use reported from these two studies indicate that device use has continued to increase over the last 5 years for most individuals and compared to 1 year ago may be plateauing or still increasing. The total hours of ED use from the EDUQ were also similar between the studies with Australian participants in the cross-sectional study reporting a mean ± SD of 9.1 ± 3.1 hours/day, and EDUQ development study 8.9 ± 3.2 hours/day (reported in EDUQ1).

Recalling that the factors determining severity of photochemical damage from BL at the macula are intensity and time of light exposure, several hypotheses emerge from the thesis outcomes. One hypothesis is that no relationship between MPOD and ED use was present as the threshold of damaging exposure has not yet been met. Alternatively, the threshold is only just being met with the relative recency of more ED use and more intense light emitting diodes being utilised in the EDs.

[172, 174] Thus, the impacts of BL exposure from EDs were not yet observable through the low EDUQ tool validity and MPOD. Another possibility is that the method of HFP used was not able to detect any changes in MPOD. The MPS II does not provide spatial distribution of the macular pigments, it is a comparison of 1 degree eccentricity to 8 degrees eccentricity. [29, 51] It may be that a different HFP method, or other measure of MPOD that is able to provide spatial distribution of the macular pigment may have shown changes. That is, perhaps MPOD changes are occurring at 2 degrees eccentricity and are thus missed by the MPS II method.

As previously explained (section 4.5), in addition to the spatial distribution of MPOD, consideration must also be given to the idea that MPOD may not be an appropriate surrogate marker of BL induced macular damage. Lutein and Z can act in multiple ways to mitigate potential damage from BL exposure such as BL absorption, indirect antioxidant activity and direct antioxidant activity. Therefore, it may be that L/Z act sparingly as an electron donor and as such, an observable shift in MPOD is not present. It may be that oxidative stress as a result of ED BL exposure is occurring without observable impact on MPOD, and any increased risk of AMD may be slow and cumulative over the lifetime. Oxidative stress is proposed to contribute to the development of other documented indicators of AMD risk such as drusen. [231] Therefore, future research may consider measurement of other ocular markers such as drusen.

The outcomes determined through these two studies contribute to addressing the thesis aims (Figure 6-2, page 163). This outcome is that BL exposure from EDs does not appear to be negatively associated with MPOD, and thus may not be a confounding factor when attempting to relate dietary L/Z intake to MPOD. In relation to the nine criteria (Figure 6-1, page 161), ED use is not currently a measure of interest when assessing the evidence base for criterion 6 or criterion 4. However, it must also be considered a longitudinal study design may be needed to capture any slow and cumulative impacts of chronic BL exposure from EDs. Therefore, further research is needed to improve the validity of the EDUQ and confirm the role of ED BL exposure in macular health. A larger sample size with more diverse patterns in daily ED use and dietary L/Z intake may also be needed to allow for demonstration of any potential relationship between ED BL exposure and MPOD. Future research should also consider measuring an additional or different surrogate marker of macular health such as drusen, or look to monitor ED use in cohort studies investigating the incidence of AMD.

6.4 Barrier 3: Minimal data for lutein and zeaxanthin in Australian food composition tables

To highlight the importance of relevant food composition values and the work conducted as part of Chapter 5 this section of the discussion presents new information not previously mentioned in the thesis. This section presents the results of Chapter 2 and Chapter 4 re-analysed with dietary L/Z intake outcomes calculated using the L/Z food composition values substituted from the foods of Chapter 5 and available FSANZ values.

The lack of comprehensive local FCTs for L/Z in many countries was identified as a barrier to meeting criterion 3 of the thesis research framework (Figure 6-2, page 163). Access to relevant FCTs being necessary to determine dietary recommendations was supported by a more recent framework for developing recommended intakes of dietary constituents with biological activity. [4] This four-step framework relies on each step being met sequentially. Having a relevant food composition database for the constituent of interest is positioned within step 1. Therefore, in relation to the outcomes of this thesis, even with an alternative framework, L/Z would still not have met the criteria to determine a recommendation for dietary intake in Australia or UK. In addition to the minimal FCT data identified in this thesis, the method inaccessibility or frequent use of non-L/Z specific methods in values reported in the databases was flagged as a barrier to meeting criterion 3 (Figure 6-1, page 161). The issues surrounding Australian local FCT data were explored in the food composition study (Chapter 5) and partially in the screener development study (Chapter 2).

The food composition study addressed the fourth thesis objective. This objective looked to investigating an appropriate extraction method for analysing food L and Z concentrations to expand the Australian FCTs. The study outcomes highlighted that while a reliable analysis method for L/Z is available, therefore meeting criterion 2 (Figure 6-1, page 161), continual testing and specialisation of a method is needed. The specialisation of the method is needed to optimise it for each individual food. For example, method variation 9 was optimal for both L and Z for broccolini but only L for baby spinach (Table 5-7, Table 5-8). Additionally, the method needs to be specialised to meet the needs of what the data will be used for, such as a FCT. For example, prioritising food sampling methods that are representative of the available food supply, extracting only edible components of the food, and preparing the food in the form that it would be consumed (e.g. cooked versus raw). Another important aspect of this study was the inability to explore whether the extraction methods used may have accounted for the differences in food L/Z concentrations observed compared to FSANZ reported values. This was unable to be explored as the methods used in the data reported by FSANZ are not openly accessible. This thesis importantly contributes to the justification for ensuring methods used for L/Z extraction from foods are openly accessible so the

evidence base relevant to criterion 3 can be improved. Method availability ensures differences in measured L/Z concentrations between studies, laboratories, and countries can be explored and accounted for in comparisons.

The results of the food composition study also make clear the importance of FCT data that is representative of the food available to the population of interest. Data that is not representative of the food supply local to the population of interest may bring error to the investigation of more complex relationships. For example, associations between dietary L/Z intake, blood L/Z, MPOD, and risk of AMD are impacted by data that is inaccurate for the local food supply. Food composition data underpins all research regarding dietary L/Z intake. Thus, meeting criterion 3 plays an important role in being able to strongly address all other criteria. The need to meet criterion 3 with local FCTs can be exemplified by substituting the food composition data obtained in this thesis work when calculating the dietary intake for the screener validation and cross-sectional studies.

Table 6-3 Chapter 2 screener development study daily milligrams of lutein and zeaxanthin intake calculated from the USDA, thesis food composition analysis, or FSANZ values

	Chapter 2 original	Values substituted from study 5	Values substituted from FSANZ	Difference: Original minus study 5	Difference: Original minus FSANZ	Difference: FSANZ minus study 5
MS1						
Mdn (25^{th} – 75^{th} %ile)	3.3 (2.2 – 5.3) [a]	3.4 (2.4 – 5.8) [b]	3.2 (2.1 – 5.3) [c]	-0.21 (-0.57 – -0.01)	0.07 (0.03 – 0.21)	-0.36 (-0.74 – -0.10)
Min	0.5	0.5	0.4	-2.69	-0.02	-3.29
Max	10.7	12.6	10.1	0.21	0.57	0.12
MS2						
Mdn (25^{th} – 75^{th} %ile)	2.7 (1.7 – 3.5) [a]	2.9 (1.8 – 3.9) [b]	2.6 (1.7 – 3.5) [c]	-0.13 (-0.33 – 0.02)	0.07 (0.01 – 0.16)	-0.25 (-0.42 – -0.01)
Min	0.7	0.7	0.7	-1.22	-0.01	-1.39
Max	9.0	9.5	8.7	0.63	0.59	0.35
4 CWS						
Mdn (25^{th} – 75^{th} %ile)	2.8 (2.1 – 4.3) [a]	3.5 (2.1 – 4.6) [b]	2.8 (1.9 – 3.9) [a, b]	-0.27 (-0.55 – -0.03)	0.08 (0.01 – 0.16)	-0.30 (-0.68 – -0.08)
Min	0.8	0.8	0.7	-1.83	-0.02	-1.87
Max	9.0	10.7	8.9	0.16	0.44	0.02

Data reported in mg/day of lutein and zeaxanthin intake. Chapter 2 original refers to dietary L/Z intake outcomes reported in Chapter 2 analysed using the USDA tables only. Study 5 refers to the food composition study (Chapter 5). Different superscript letters within a row indicates significant difference $p<0.001$ analysed by Related-Samples Wilcoxon Signed Rank Test.
Abbreviations: MS1, monthly screener 1; mdn, median; %ile, percentile; min, minimum; max, maximum; MS2, monthly screener 2; CWS combined weekly screeners; FSANZ, Food Standards Australia New Zealand.

Differences in daily L/Z intake from the monthly and weekly screeners in the screener development study when specific L/Z values were substituted for different reference values is demonstrated in Table 6-3. Keeping all other values from the USDA database, the raw baby spinach and cooked broccoli values were substituted with the thesis food composition analysis data. Only baby spinach and broccoli were used as the screener did not include dried goji berries, baby orange capsicum, or broccolini. Similarly, keeping all other values from the USDA database, available FSANZ L values were substituted. FSANZ values were available for raw strawberries, cooked orange carrot, cooked green peas, cooked egg, and cheddar cheese. Substitution with the thesis food composition analysis study values resulted in a statistically significant difference in median daily L/Z intake compared with the original analysis for all screeners using only the USDA tables. Substitution with the FSANZ values also resulted in significant differences for both monthly screeners data. The difference between medians of the original and thesis food composition study substituted values, although statistically significant, is only 0.21 mg/day. However, in the context of existing research, this may be a meaningful difference as increases in MPOD have been reported over 6 months with daily avocado consumption that provided only 0.5 mg/day L. [37] It is important to note the food absences from the L/Z screeners. In the development of the L/Z screeners, foods such as broccolini, baby orange capsicum, and dried goji berries were omitted due to no USDA L/Z values being available for them. As demonstrated in the food composition analysis study, all three of these foods are available in the Australian food supply and contain L/Z. Omission of these foods from the screener suggests reported intake in this thesis is systematically lower that true intake. This thesis finding has implications for prior research in which foods such as goji berries are available in the food supply but dietary analysis has relied upon the USDA FCTs. In such studies, dietary L/Z intake may be systematically lower that true intake as contributions of the goji berries are missed. The difference in L/Z daily intake found when either the thesis food composition data or FSANZ values were substituted suggests outcomes from studies such as the Australian Blue Mountains Eye Study may have differed if local FCTs had been available and used. [13] Comprehensive local food supply composition data would allow for inclusion of foods such as goji berries within the screener. Inclusion of relevant foods is necessary to investigate the relationship most accurately between dietary L/Z, plasma L/Z and MPOD.

Table 6-4 Chapter 4 cross-sectional study monthly screener daily milligrams of lutein and zeaxanthin intake calculated with USDA, thesis food composition analysis, or FSANZ values

	Chapter 4 original	Values substituted from study 5	Values substituted from FSANZ
Mdn (25^{th} – 75^{th} %ile)	4.6 (2.7 – 7.4) [a]	4.1 (2.3 – 6.4) [b]	3.4 (2.0 – 5.5) [c]
Min	0.4	0.4	0.4
Max	22.4	22.5	21.9

Chapter 4 original refers to dietary L/Z intake outcomes reported in Chapter 4 analysed using the USDA tables only. Study 5 refers to the food composition study (Chapter 5). Abbreviations: FSANZ, Food Standards Australia New Zealand; mdn, median; %ile, percentile; min, minimum; max, maximum. All data reported in mg/day. Different superscript letters within a row indicates significant difference $p<0.001$ analysed by Related-Samples Wilcoxon Signed Rank Test.

The impact of FCT data on these more complex relationships is exemplified with the substitution of the thesis food composition analysis data or the FSANZ food composition L/Z values into the cross-sectional study MS (Table 6-4). The L/Z intake from the MS in the cross-sectional study was 4.6 mg/day with the original analysis reliant only on the USDA tables. This intake was significantly different to the 4.1 mg/day from the thesis food composition substituted values, and 3.4 mg/day from FSANZ substituted values ($p<0.001$). There were also differences in the strength of individual Spearman correlations of MS dietary intake with MPOD and plasma L/Z (Table 6-5). The relationship between dietary L/Z intake and MPOD strengthened marginally but remained non-significant with either of the substituted values. The relationship between dietary intake and combined plasma L/Z remained significant but was weaker with the thesis food composition analysis study substituted values, and stronger with FSANZ values. Using the thesis composition analysis or FSANZ substituted values, the MS total intake values did not significantly change the multiple linear regression model outcomes observed in the cross-sectional study (Chapter 5).

Table 6-5 Chapter 4 cross-sectional study associations between dietary intake and MPOD or plasma lutein and zeaxanthin with substituted food composition values

	MPOD			Plasma L/Z		
	r	R^2	p	r	R^2	p
Chapter 4 original MS L/Z intake	0.090	0.023	0.38	0.283	0.349	0.008
MS L/Z intake values substituted from Study 5	0.105	0.027	0.31	0.276	0.315	0.010
MS L/Z intake values substituted from FSANZ	0.110	0.027	0.29	0.300	0.361	0.005

Chapter 4 original refers to dietary L/Z intake outcomes reported in Chapter 4 analysed using the USDA tables only. Study 5 refers to the food composition study (Chapter 5). Abbreviations: MS, monthly screener; L/Z, lutein and zeaxanthin; FSANZ, Food Standards Australia New Zealand; MPOD, macular pigment optical density; r, correlation coefficient; R^2, deattenuated correlation coefficient. Two-tailed Spearman's rho correlation.

The thesis food composition analysis study substitutions were two moderate to high concentration L/Z foods (803 μg/100g and 9189 μg/100g); cooked broccoli and baby spinach. The FSANZ substitutions were two foods with moderate L/Z concentrations (300 – 620 μg/100g), and three low concentration foods (<100 μg/100g). [229] The differences observed with the substituted values highlight the possible bias with error from FCTs. The differences in L/Z food concentrations measured in the food composition study, and how these values impacted the screener development and cross-sectional study dietary L/Z intakes are important contributions of this thesis. They are important as they provide strong justification that the differences between USDA and non-US FCTs exist and have an impact. Thus, this thesis indicates criterion 3 of the research framework is not met in Australia. Additionally, it is unlikely that criterion 3 is able to be met in other non-US countries reliant on the USDA tables for dietary L/Z estimation. Minimal local FCT for L/Z is a barrier to meeting criterion 3 and the other criteria that are reliant on FCTs to achieve research outcomes, for example criterion 4. Development of local L/Z FCTs is needed to meet criterion 3 and strengthen the research to support related criteria. In relation to the overall thesis research question, local food composition data analysed with methods specific to the L/Z and the foods of interest are needed when attempting to capture habitual dietary L/Z intake in a quantitative and valid manner.

6.5 Implications for a dietary lutein and zeaxanthin intake target value

The outcomes of this thesis indicate that globally, and in Australia and the UK specifically, the evidence base does not currently meet all nine criteria for L/Z to be constituents with dietary intake targets. The evidence base is not yet adequate as habitual dietary L/Z intake cannot yet be validly and quantitatively captured, and in non-US context food composition tables my not be representative of the local food supply. The outcomes of this work have addressed gaps and highlighted remaining gaps in the research for criteria 2, 3, 4, and 6 (Figure 6-1, page 161).

Criterion 2 is a reliable analysis method. Specific to food composition data, this thesis confirmed that a reliable analysis method can be determined. However, it is important to note that previous methods used to generate FCT data are often conducted with assays that were not optimised for L/Z measurement specifically. [260] This may mean the data presently available in many FCTs is not closely representative of the L/Z available in the food supply. Additionally, detail on the analysis methods used to quantify L/Z, such as for the FSANZ FCTs, are not always available. [229] Therefore, it is not possible to determine whether differences between older and newer data are related to analysis methods, cultivars, pre- and post-harvest factors, or a combination of these. The outcomes of the food composition study indicate that representative food sampling and use of food-

specific optimised L/Z extraction methods are needed to generate data appropriately reliable for use in FCTs.

Criterion 3 refers to a food database with known amounts of the bioactive constituent. It is indisputable that in both the UK and Australia there is not currently adequate local L/Z food composition data points to meet criterion 3. [171, 229] As previously outlined (Section 6.4), using non-local tables, such as using the USDA tables in an Australian population, is not optimal due the observed differences in food L/Z concentrations between countries. [138] While the USDA FCTs are large and can capture the majority of L/Z from dietary intake, the outcomes of this thesis suggest criterion 3 may not be met even in the US context. The data may not be comprehensive enough as it is missing entries for some moderate to high L/Z concentration foods including broccolini, orange capsicum, and dried goji berries. Additionally, the aforementioned importance of optimising the L/Z extraction method for each food individually suggests entries in the USDA FCTs, that were reliant on methods not specific to L/Z, may not be representative of true L/Z concentrations. Further research of local US based foods would be needed to confirm whether the USDA FCTs are representative of the current food supply. Despite these questions, the USDA FCTs are the most comprehensive and available tables applicable to Western-influenced dietary intake. [138] In Australia and the UK, without an understanding of the L/Z available in the food supply, criterion 3 is not met and a target intake for L/Z cannot be determined.

Criterion 4 refers to cohort studies. Several outcomes of this thesis suggest that the outcomes of past cohort studies investigating dietary L/Z intake and MPOD status or risk of AMD should be interpreted with caution. One of these outcomes was the finding from the narrative literature review that no dietary intake tools used in these studies have been specifically validated to capture dietary L/Z intake. [67] Another outcome observed was the poor validity of the monthly and weekly screeners (Chapter 2). The inability to capture habitual dietary L/Z intake with a screener specifically designed for L/Z suggests prior research that has relied on less specific tools was likely inaccurately capturing L/Z intake. The impact of a tool with poor validity was highlighted in the cross-sectional study (Chapter 4). Dietary L/Z intake from the MS was not correlated with MPOD and only weakly correlated with plasma L/Z concentrations. As previously discussed, the validity of dietary L/Z is also influenced by the FCTs referenced. Therefore, outcomes of prior cohort studies also using tools with poor validity and attempting to relate dietary L/Z intake to variables such as MPOD or risk of AMD should be interpreted with caution. Whilst there is undoubtably a relationship between L/Z intake and MPOD [33], the degree to which habitual dietary intake relates

to risk of AMD remains unclear. Further cohort studies investigating the relationship between dietary L/Z intake using a valid tool, and macular health are needed to meet criterion 4.

Criterion 6 is about clinical trials demonstrating dose-response and efficacy. To determine a recommendation or target for daily L/Z intake, a dose-response relationship and the demonstration of the efficacy of a target LZ intake on a biomarker must be known. Research to date has been heavily focussed on the dose-response relationship with supplemental intake and MPOD or AMD risk and progression. As previously identified (section 1.3), fewer studies exist investigating the dose-response relationship between dietary L/Z interventions and MPOD. Most importantly, inclusive of the studies in this thesis, no studies exist that have used a dietary intake questionnaire validated to capture habitual dietary L/Z intake and relate it to MPOD or AMD risk. A dose-response relationship between dietary L/Z intake and MPOD or AMD risk has yet to be determined. Globally, criterion 6 cannot currently be confidently met, and thus a target for habitual dietary L/Z intake cannot yet be determined. Improvement to the validity of methods available to capture habitual dietary L/Z intake, such as the screeners investigated in this thesis, is needed to progress research relating to criterion 6.

The outcomes of this thesis indicate researchers and professionals providing nutrition advice surrounding L/Z should be aware of the current limitations of attempting to estimate habitual dietary L/Z intake, and lack of local food composition tables in locations such as Australia. This limitation means professionals should be critically interpretating the results of past and future research. The thesis outcomes also provided an indication of key foods to be cognisant of when continuing research or practice to estimate dietary L/Z intake. Foods that commonly contributed to intake of L/Z in this thesis in Australian and UK individuals were baby spinach, broccoli, pumpkin, zucchini, orange carrot, and egg.

6.6 Strengths and limitations

6.6.1 Strengths of the thesis

This thesis is the first to develop and investigate the validity of a dietary screener designed specifically to quantitatively capture habitual dietary L/Z intake. The development of this screener has made a significant contribution to the field of nutrition research. The screener validation process has highlighted the difficulties present when attempting to capture a valid habitual dietary L/Z intake. This presents as a major barrier in determining dietary intake targets because it prevents accurate determination of key relationships such as dietary L/Z dose-response with MPOD. Another

strength of this thesis is the finding that use of different extraction methods resulted in significant differences in measured L/Z concentrations in foods. This finding reaffirms the importance of open access sharing of methods to increase consistency in optimisation of results. Extraction methods utilised for the data present in the Australian and USDA FCTs were not always accessible. Thus, the role of the extraction method was unable to be explored in the notable differences found between the USDA, the Australian and this thesis data about LZ food concentration. These notable differences also strengthen the justification for local FCTs, specifically expanding on available Australian food composition data.

This thesis was also the first to develop and investigate the validity of a questionnaire designed to capture habitual ED use, the EDUQ. With improvements to validity, this novel questionnaire has applicability in the field of macular health, including the investigation of AMD pathology. This questionnaire also has potential applicability in other fields of research such as sleep and sedentary behaviour, or in disciplines of psychology. [214, 215] For example, emerging areas of research investigating relationships between consumption of media through EDs and conditions such as depression and body dissatisfaction could be applications of the EDUQ. [275] In this thesis the EDUQ was used to investigate for the first time whether a relationship between ED BL exposure and MPOD exists in humans. This investigation is highly valuable as the outcomes suggest that ED BL exposure may not be an environmental risk factor for AMD. However, due to the poor validity of the EDUQ found as a part of this thesis, continued research is needed to confirm this finding. Improved tool validity and investigations in more diverse populations are needed to improve future research outcomes.

6.6.2 Limitations of the thesis

A limitation of this thesis was the participant characteristics of study populations recruited in the two validation studies and the cross-sectional study (Chapter 2, 3, and 4). The participant characteristics across all three studies were predominantly female, below 40 years of age and tertiary educated. This lack of participant diversity means the findings may not be applicable to the general Australian or UK populations. The degree to which the findings are not applicable is unclear. It is unclear in the literature how dietary L/Z intake and ED use behaviours differ amongst different Australian and UK population groups. Despite this, from broader understandings of dietary patterns and daily activities it would be reasonable to expect poor generalisability of the thesis findings. For example, the percentage of Australian adults reported to be meeting daily vegetable intake recommendations differs between age groups. National data from 2014-15 indicated only 3.7% of adults 18–24 years were meeting five serves of vegetables per day compared

to 10.9% of adults 65–74 years. [276] Similarly, it is likely that ED use patterns would differ between individuals of different age, sex, or education status.

Another limitation of this thesis was the reliance on the monthly L/Z screener and EDUQ tools in the cross-sectional study (Chapter 4). These two tools were found to have poor validity as part of this thesis. Therefore, the findings of the cross-sectional study must be interpreted with caution despite the novel contribution of investigating potential relationships between MPOD, dietary L/Z and ED BL exposure.

6.7 Conclusions

Thesis objectives one and two were both partially met with the successful development of the MS, WS, and EDUQ. The goal within objectives one and two to validate these tools was not met. Objective three was met, with outcomes suggesting usual ED use is not presently associated with MPOD. However, as the ED use tool demonstrated poor validity, additional research is needed to confirm this outcome. Objective four was also met with an extraction method to analyse L and Z concentrations for use in a FCT determined for the five investigated foods. This thesis has contributed three novel tools, examined a food composition extraction method, and demonstrated notable between-country differences in food composition data.

In addressing the thesis objectives, the primary research question of this thesis, 'How can habitual dietary L and Z intake be validly and quantitatively estimated to investigate links to ocular health?', has been partially answered. This thesis has indicated that valid estimation of habitual dietary L/Z measurement is very difficult and not yet possible. However, the key factors that must be addressed to achieve measurement (the 'how') were identified. One factor is having available local FCTs, for example in Australia. Other factors include having a dietary intake tool that is minimally impacted by non-ubiquitous food L/Z distribution, memory recall bias, and can capture intake over a timeframe that is reflective of L/Z plasma and MPOD turnover.

The screener development study highlighted the importance of appropriate statistical methods for tool validation, specifically the potential overestimation of questionnaire validity with correlational statistics compared to a Bland-Altman plot analysis. The screener development study also highlighted a small subset of food that contributed notably to total dietary L/intake. These outcomes can be used to conduct further research to improve the validity of the screener or a similar tool.

This thesis has also confirmed that without a valid quantitative tool, the relationship between dietary L/Z intake and biological markers such as plasma L/Z and MPOD cannot be clearly interpreted. The impacts of dietary L/Z intake may be weaker than prior research suggests; it is also biologically plausible that the impacts are stronger than prior research has been able to demonstrate. The thesis findings strongly contribute to an understanding that the evidence base does not yet

support determination of a target dietary intake for L/Z. However, the findings of this thesis also provide direction on how to improve the validity of quantitative dietary L/Z intake estimation, and subsequently strengthen the evidence base to support a dietary target for L/Z.

Chapter 7 Future Directions

The findings of this thesis support the pursuit of multiple research avenues. These research avenues relate to strengthening the evidence base to support understanding of the role of dietary L/Z intake in ocular health conditions such as AMD. Some avenues would also support alternate fields of research such as psychology.

7.1 Local food composition tables

The first research avenue is the development of local FCTs for L/Z. The outcomes of this thesis provide demonstrated differences in Australian food L/Z concentrations with that reported in the USDA FCTs. [138] The identification of these differences supports the development of local L/Z FCTs. Recommendations and key considerations from this thesis for future L/Z food composition analysis include:

- A single extraction method may be appropriate for use across a wide array of foods however, preliminary testing to ensure optimisation of the method is needed for each food individually.
- High biological variability in L/Z concentrations are present within and between foods. Food sampling that is representative of the food supply for the population of interest is of high importance for developing FCTs.
- Separation of L and Z in analysis is important as their ratios between foods are variable. Additionally, individual values for L and Z would allow for FCT data to support investigation of the individual role dietary L and Z play in conditions such as AMD, and potentially individual dietary target values.

7.2 Measurement of habitual dietary lutein and zeaxanthin intake

The second research avenue is improving the validity of tools used to capture habitual dietary L/Z intake. Tool improvement would support research into the dose response relationship between L/Z intake and MPOD as a surrogate marker of AMD risk. Avenues to improve the validity of dietary L/Z intake capture include developing an in-depth understanding of patterns of dietary L/Z intake. Such an understanding would make clear the number of repeat captures required by a tool, such as a 24-hour diet recall, to capture habitual dietary L/Z intake. Alternatively, tools such as the monthly and weekly screeners developed as part of Chapter 2 could look to be modified to improved validity. Recommendations to improve the validity of the dietary L/Z screeners include:

- Follow up questions regarding high contribution L/Z foods to assist more thoughtful recall of these foods from respondents.

- Visual aids, such as pictures of serve sizes or a photographic atlas, to assist respondent estimation of intake. [91]
- Food composition tables local to the respondents of interest to improve that accuracy of quantitative values obtained. [4]
- Development of a mini dietary L/Z screener that only lists foods contributing substantially to intake in the respondent population of interest, for example baby spinach as identified in the participant groups of this thesis.
- Availability and utilisation of an objective measure, such as a biological marker, that is representative of habitual dietary L/Z intake. A biological marker, such as blood L/Z concentrations, are valuable in the validation process of a dietary intake method, and as a method to screen for accuracy of respondent reporting.

It is important to note that a protocol to capture an objective measure that is reflective of habitual dietary intake has not yet been determined. A robust biochemical marker would improve dietary intake research through a greater potential to determine and address reasons for poor dietary tool validity. Blood L/Z is a preferrable biomarker, however an improved understanding of blood L/Z half-life, and mechanisms of uptake and release of L/Z from other tissues such as adipose tissue is needed.

7.3 Measurement of electronic device use

The third research avenue is continued investigation and monitoring of ED use, such as with the EDUQ. The monitoring of ED use may have application in exploring the role of BL in macular conditions such as AMD. However, the capture of ED use may also have application in understanding other ocular conditions such as computer vision syndrome and myopia. [204, 221] Specific to the investigation of ED use and macular health recommendations for future research include:

- Targeted study recruitment of individuals with diverse ED use behaviours, specifically low and high use. In comparison to the study populations of this thesis, this targeted recruitment would likely look like greater diversity in participant, age, sex, and occupational status.
- Capture of other markers of macular health in addition to MPOD such as drusen deposits.

In addition to ocular health, the EDUQ may have application in other research fields such as sleep, physical activity, musculoskeletal disorders, and psychology. [202, 214, 215] The application within psychology may include investigation of depression and body dissatisfaction. To effectively apply the EDUQ in these areas the validity of the tool may require improvement. Recommendations from this thesis to improve the validity of the EDUQ include:

- Ensuring validity of the tool in the study population of interest.
- Although it does not exclusively capture ED use, the utilisation of an objective measure such as Clouclip and RangeLife glasses. [204, 205] Alternatively, use of applications such as RealizD that record when devices are in use. [202]
- If using the 24-hour device use diary as a comparative tool, modify the reporting interval of the EDUQ to be 15-minutes so it is aligned with the reporting interval of the 24-hour device use diary.
- In addition to, or in place of the 24-hour device use diary, utilise an alternate method to test relative validity of the EDUQ such as a 3-day device use diary or direct observation.

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Chapter 9 Appendices

Appendix A: Ethical approvals

A-1 Validation of two lutein and zeaxanthin intake questionnaires, and an electronic device use questionnaire, University of Queensland Health and Behavioural Sciences, Low and Negligible Risk Ethics Sub-Committee (2020001774)

THE UNIVERSITY OF QUEENSLAND

Institutional Human Research Ethics Approval

Project Title:	Validation of two lutein and zeaxanthin intake questionnaires, and an electronic device use questionnaire
Chief Investigator:	Dr Veronique Chachay
Supervisor:	Dr Veronique Chachay
Co-Investigator(s):	Ms Naomi Fitzpatrick, Dr David Briskey, Prof Sandra Capra, Prof Joanna Bowtell, Dr Sarah Jackman, Prof Angela Shore
School(s):	School of Human Movement and Nutrition Sciences, The University of Queensland
Approval Number:	2020001764
Granting Agency/Degree:	PhD
Duration:	30 September 2022

Comments/Conditions:

- HREA Form, 24/07/2020
- EDUQ Recruitment Video, 24/07/2020
- EDUQValidation_Participation Withdrawal Form_Fitzpatrick_V1, 24/07/2020
- EDUQValidation_PIF_Fitzpatrick_V2, 06/08/2020
- FFQ Recruitment Video, 24/07/2020
- FFQEDUQ20_Protocol_Fitzpatrick_V1, 24/07/2020
- FFQValidation_Participation Withdrawal Form_Fitzpatrick_V1, 24/07/2020
- FFQValidation_PIF_Fitzpatrick_V2, 06/08/2020

Note: if this approval is for amendments to an already approved protocol for which a UQ Clinical Trials Protection/Insurance Form was originally submitted, then the researchers must directly notify the UQ Insurance Office of any changes to that Form and Participant Information Sheets & Consent Forms as a result of the amendments, before action.

Name of responsible Sub-Committee:
University of Queensland Health and Behavioural Sciences, Low & Negligible Risk Ethics Sub-Committee
This project complies with the provisions contained in the *National Statement on Ethical Conduct in Human Research* and complies with the regulations governing experimentation on humans.

Name of Ethics Sub-Committee representative:
Professor Bill von Hippel
University of Queensland Health and Behavioural Sciences, Low & Negligible Risk Ethics Sub-Committee

Signature _____ Date 17/08/2020

A-2 Validation of two lutein and zeaxanthin intake questionnaires, and an electronic device use questionnaire, University of Queensland Health and Behavioural Sciences, Low and Negligible Risk Ethics Sub-Committee (2020001774) – Amendment

THE UNIVERSITY OF QUEENSLAND
AUSTRALIA
CREATE CHANGE
Research Ethics and Integrity

Human Research Ethics Approval

Project Number:	2020/HE001764
Project Title:	Validation of two lutein and zeaxanthin intake questionnaires, and an electronic device use questionnaire.
Version:	2.02
Chief Investigator:	Dr Veronique Chachay School of Human Movement and Nutrition Sciences
Co-Investigator(s)	Prof Angela Shore Dr David Ronald Briskey Prof Joanna Bowtell Naomi Fitzpatrick Emeritus Professor Sandra Maureen Capra Dr Sarah Jackman
Funding Body (UQ ref#):	
Approving Committee:	HABS LNR
Approval End Date:	30 Sep 2022
Date of Approval:	Wednesday, 7 July 2021

HABS LNR confirms that this project meets the requirements of the National Statement on Ethical Conduct in Human Research (2007, current revision). The University's human research ethics committees are organised and operate in accordance with the National Statement on Ethical Conduct in Human Research (2007, current revision).

Approved Documents

Document Type	File Name	Document Tile	Application Version	Document Version	Last Modified
Project Protocol	2020_HE001764_FFQEDUQ20_Protocol_V3_clean.docx.docx	2020_HE001764_FFQEDUQ20_Protocol_V3_clean.docx.docx	2.2	2	6/07/2021 10:08:33 PM
Change Tracking	2020_HE001764 v2_01 - v2_02 Changes.pdf	2020/HE001764 v2_01 - v2_02 Changes	2.2	1	6/07/2021 10:08:36 PM
Application	Output Form.pdf	Output Form	2.2	2	6/07/2021 10:08:32 PM

Application Attachment	2020_HE001764_UK EDUQ_PIF.doc	UK version of participant information form for EDUQ option.	2.2	1	6/07/2021 10:08:33 PM
Application Attachment	2020_HE001764_UK FFQ_PIF.docx	UK version of participant information form for FFQ option.	2.2	1	6/07/2021 10:08:34 PM
Application Attachment	2020_HE001764_UK EDUQ_Participation Withdrawal Formdocx.docx	UK version of participation withdrawal form for EDUQ option.	2.2	1	6/07/2021 10:08:33 PM
Application Attachment	2020_HE001764_UK FFQ_Participation Withdrawal Form.docx	UK version of participation withdrawal form for FFQ option.	2.2	1	6/07/2021 10:08:34 PM
Application Attachment	2020_HE001764_FFQEDUQ20_Protocol_V3_tracked changes.docx	Version 3 of Protocol document with tracked changes.	2.2	1	6/07/2021 10:08:33 PM

Ethics committee representative

Jolanda Jetten
Chair
HABS LNR
The University of Queensland

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A-3, Investigating associations between chronic electronic device blue light exposure, dietary xanthophylls intake and macular pigment density in humans. University of Queensland Research Ethics Committee A (2019002736)

THE UNIVERSITY OF QUEENSLAND

Institutional Human Research Ethics Approval

Project Title:	Investigating associations between chronic electronic device blue light exposure, dietary xanthophylls intake and macular pigment density in humans.
Chief Investigator:	Naomi Fitzpatrick, Dr Veronique Chachay
Supervisor:	Dr Veronique Chachay, Dr David Briskey, Prof Sandra Capra, Prof Joanna Bowtell, Prof- Angela Shore, Dr Sarah Jackman
Co-Investigator(s):	Dr David Briskey, Prof Sandra Capra, Prof Joanna Bowtell, Prof- Angela Shore, Dr Sarah Jackman
School(s):	School of Human Movement and Nutrition Sciences
Approval Number:	2019002736
Granting Agency/Degree:	PhD
Duration:	28 February 2022

Comments/Conditions:

1-NF00043_Output Form
2-NF00043_Lutein and MPOD_Protocol__Version 2_
3-NF00043_Lutein and MPOD_PICF_Version 2
4-NF00043_Blue Light Questionnaire (EDUQ)_Fitzpatrick_
5-NF00043_Lutein_Participation Withdrawal Form_Fitzpatrick_V1
6-NF00043_LZ-FFQ_Fitzpatrick
7-NF00043_Study Ad Lutein-Fitzpatrick_Cross Sectional
8-NF00043_signature
9-CI response to Cmt feedback

Note: if this approval is for amendments to an already approved protocol for which a UQ Clinical Trials Protection/Insurance Form was originally submitted, then the researchers must directly notify the UQ Insurance Office of any changes to that Form and Participant Information Sheets & Consent Forms as a result of the amendments, before action.

Name of responsible Committee:
University of Queensland Human Research Ethics Committee A
This project complies with the provisions contained in the *National Statement on Ethical Conduct in Human Research* and complies with the regulations governing experimentation on humans.

Name of Ethics Committee representative:
Dr Gordon McGurk
Chairperson
University of Queensland Human Research Ethics Committee A
Registration: EC00456

Signature _____ Date **28/02/2020**

A-4, Investigating associations between chronic electronic device blue light exposure, dietary xanthophylls intake and macular pigment density in humans. University of Queensland Research Ethics Committee A (2019002736) – Amendment 07/09/2020

THE UNIVERSITY OF QUEENSLAND

Institutional Human Research Ethics Approval

Project Title:	Investigating associations between chronic electronic device blue light exposure, dietary xanthophylls intake and macular pigment density in humans – 21/08/2020 - AMENDMENT
Chief Investigator:	Naomi Fitzpatrick, Dr Veronique Chachay
Supervisor:	Dr Veronique Chachay, Dr David Briskey, Prof Sandra Capra, Prof Joanna Bowtell, Prof- Angela Shore, Dr Sarah Jackman
Co-Investigator(s):	Dr David Briskey, Prof Sandra Capra, Prof Joanna Bowtell, Prof- Angela Shore, Dr Sarah Jackman
School(s):	School of Human Movement and Nutrition Sciences
Approval Number:	2019002736
Granting Agency/Degree:	PhD
Duration:	28 February 2022

Comments/Conditions:

Amendment Form, 21/08/2020
NF00043_Blue Light Questionnaire (EDUQ)_Fitzpatrick__v3_clean
NF00043_Blue Light Questionnaire (EDUQ)_Fitzpatrick__v3_tracked changes
NF00043_Cross-sectional study recruitment video
NF00043_Lutein_Cross Sectional Study_QML Consent Form_v3
NF00043_Lutein_Protocol_Fitzpatrick_v3_clean
NF00043_Lutein_Protocol_Fitzpatrick_v3_track changes

Note: if this approval is for amendments to an already approved protocol for which a UQ Clinical Trials Protection/Insurance Form was originally submitted, then the researchers must directly notify the UQ Insurance Office of any changes to that Form and Participant Information Sheets & Consent Forms as a result of the amendments, before action.

Name of responsible Committee:
University of Queensland Human Research Ethics Committee A
This project complies with the provisions contained in the *National Statement on Ethical Conduct in Human Research* and complies with the regulations governing experimentation on humans.

Name of Ethics Committee representative:
Dr Gordon McGurk
Chairperson
University of Queensland Human Research Ethics Committee A
Registration: EC00456

Signature Gordon B J McGurk Date **07/09/2020**

Appendix B

Appendix B-1: Monthly lutein and zeaxanthin screener

Monthly Screener Questionnaire

1.1 Thinking back over the last 1 month, please indicate your usual intake of the following foods:

NOTE:

- Serves have been chosen to reflect common serve sizes of this food. You may have eaten multiple serves or less that 1 serve. To indicate less than 1 you can use fractions of serves (¼, ½, ¾) or thirds (1/3, 2/3), or numbers (0.25, 0.5, 0.75).
- All foods below are fresh or raw unless specified otherwise.

*Don't eat at all or a food you consume very rarely such as once every 6 months or less frequently.

Food	**Serve Size**	**Reflect over the last month to answer the number of serves you have eaten of the foods below.**			
		Number of serves **per week**. (If **less than 1** leave blank and answer in per month column only)	Number of **serves over last month**.	If haven't eaten a food at all in the last month please tick one of the following.	
				Do eat, but not in last month.	Don't eat at all*
Fruit					
Apple	1 apple (165g)				
Apricot	1 apricot (40g)				
Blackberries	1/3 cup (65g)				
Blueberries	¼ cup (40g)				
Blueberries, dried	1 Tbs (15g)				
Cherries, dried	1 Tbs (15g)				
Cherries, canned.	1 Tbs (20g)				
Cherries, raw	15 cherries (100g)				
Cranberries, dried	10 fruit (15g)				
Grapes (red or green)	10 grapes (50g)				
Jackfruit	¼ fruit (200g)				
Kumquats, raw	2 fruit (40g)				

Nectarines	1 nectarine (160g)				
Orange, fruit	1 medium (200g)				
Orange juice, (fresh or concentrate)	1 cup or 250ml (260g)				
Paw paw	½ medium (100g)				
Peach, dried	¼ cup (40g)				
Peach, yellow	1 peach (180g)				
Pear	1 medium (185g)				
Pear, dried	1/3 cup (65g)				
Persimmons	1 fruit (75g)				
Plum, non-native	1 plum (70g)				
Raspberries	¼ cup (35g)				
Raspberries, frozen	¼ cup (35 g)				
Strawberries	4 berries (75g)				
Vegetables:					
Artichoke	½ whole (65g)				
Asian greens, e.g. bok choy	1 cup (130g)				
Asparagus, cooked	3 spears (35g)				
Avocado	¼ avocado (40g)				
Beans, snap	5 beans (20g)				
Broccoli, cooked	4 florets (80g)				
Brussels sprouts	3 sprouts (65g)				
Cabbage, red, raw	½ cup (50g)				
Capsicum (any colour)	¼ whole (70g)				
Carrot, orange, cooked	1 medium (115g)				
Carrot, orange, raw	1 medium (125g)				
Celery	4 sticks (30g)				

Corn, sweet, yellow	½ medium cob (80g) OR ¼ cup kernels (90g)				
Cress, garden	1 cup (35g)				
Edamame	½ cup (95g)				
Fennel bulb	½ cup (75g)				
Kale	1 cup (115g)				
Kale, cooked	½ cup (60g)				
Leek, cooked	¼ cup (25g)				
Lettuce, cos or romaine	1 cup (35g)				
Okra	1 okra (10g)				
Okra, cooked	1 okra (10g)				
Olives, canned/jar	4 whole (15g)				
Pea, green, cooked	¼ cup (40g)				
Peppers, jalapeno	1 (20g)				
Pumpkin, cooked	2 medium pieces (190g)				
Rocket	½ cup (20 g)				
Rhubarb	½ stalk (75g)				
Sauerkraut	¼ cup (50g)				
Silverbeet, cooked	¾ cup (85g)				
Snacks, popcorn, air-popped	1 cup (7g)				
Spinach, baby	1 cup (45g)				
Spinach, baby, raw, cooked	1/3 cup (40g)				
Spinach, baby, frozen, cooked	1/3 cup (40g)				
Tomatoes, canned	¼ cup (60g)				
Tomatoes, sun-dried	3 slices (15g)				
Watercress	¾ cup (25g)				
Zucchini	½ zucchini (100g)				

Zucchini, cooked	½ zucchini (85g)				
Grains					
Barley, pearled, cooked	½ cup (95g)				
Bread (all types)	1 slice (40g)				
Oats	½ cup oats (uncooked) (40g)				
Pasta (wholemeal or white)	½ cup (75g)				
Rice (all types)	½ cup (100g)				
Quinoa, raw	1/3 cup (60g)				
Lean meat and poultry, fish, eggs, tofu, nuts and seeds and legumes/beans					
Beans and legumes (all types, e.g. kidney beans, lentils)	1 cup (150g)				
Egg, whole, cooked	2 (80g)				
Flaxseeds	½ Tbs (7g)				
Fish, fillet, cooked	1 fillet 100 g				
Nuts, almonds, whole	2 Tbs (30g)				
Nuts, hazelnuts, whole	2 Tbs (20g)				
Nuts, peanuts, shelled	2.5 Tbs (30g)				
Nuts, pistachios, shelled	¼ cup (30g)				
Pepitas (seeds)	1 Tbs (10g)				
Poultry, cooked	80g (e.g half breast, 1 leg)				
Read meat, cooked (all types, e.g. beef, pork, lamb)	65g (e.g. small steak, ½ cup mince)				
Tofu	3 large cubes (170g)				
Milk, yoghurt, cheese and/or their alternatives					
Milk (all types)	1 cup or 250mL				
Cheese, hard (e.g. cheddar)	2 slices (40g)				
Yoghurt	¾ cup (170g)				

Other					
Biscuits, sweet	2-3 biscuits (30g)				
Cake or muffin	1 slice or muffin (40g)				
Chocolate	4 squares or 1 small bar (30g)				
Hot chips, fried	12 chips (60g)				
Ice cream	2 scoops (75 g)				
Lollies	5-6 small (40g)				

1.2 Please indicate below any supplements you are currently taking, or have taken in the last month. (e.g. multivitamin, iron supplement)

1.3 Below are prompts to help you indicate how your dietary habits have changed over the last 10 years?

When answering the questions below please consider the following:

Fruit and vegetable intake:

- Any changes to your usual fruit and vegetable intake. This includes regularity of intake, and types of fruits and vegetables consumed. For example, compared to 1 year ago fruit intake increased from 1 serve per day of either an apple or banana, to 2 serves of fruit per day of either banana, apple, pear or blueberries.

Diets and eating patterns:

- Any diets you have followed and for how long. (Examples of diets include: low-carb diet, fasting diets such as 5:2, Lite & Easy, or ketogenic)
- Changes to eating patterns including vegetarian, vegan, gluten free, or any other food avoidances due to preference, allergy or intolerance.

Other:

- Any other factors that may have influenced your usual dietary intake. Some examples include diagnosis of medical condition (e.g. irritable bowel syndrome), or any major changes in living location that changed what foods were available to you (e.g. rural compared to urban, or living in different country).

1.3.1 How have your dietary habits changed compared to 1 year ago?

Fruit intake:

Vegetable intake:

Diets and eating patterns:

Other:

1.3.2 How have your dietary habits changed compared to 5 years ago?

Fruit intake:

Vegetable intake:

Diets and eating patterns:

Other:

1.3.3 How have your dietary habits changed compared to 10 years ago?

Fruit intake:

Vegetable intake:

Diets and eating patterns:

Other:

1.3.4 Any further notes relating to your dietary intake, or comments?

Appendix B-2: Weekly lutein and zeaxanthin screener

Weekly Screener Questionnaire

1.1 Thinking back over the last 7 days, please indicate your usual intake of the following foods:
NOTE:

- Serves have been chosen to reflect common serve sizes of this food. You may have eaten multiple serves or less that 1 serve. To indicate less than 1 you can use fractions of serves (¼, ½, ¾) or thirds (1/3, 2/3), or numbers (0.25, 0.5, 0.75).
- All foods below are fresh or raw unless specified otherwise.

*Don't eat at all or a food you consume very rarely such as once every 6 months or less frequently.

Food	**Serve Size**	**Reflect over the last 7 days to answer the number of serves you have eaten of the foods below.**		
		Number of **serves in the last week (7 days)**	If you **haven't consumed** this food at all in the last week please tick one of the following.	
			Do eat, but not in last week.	Don't eat at all*
Fruit				
Apple	1 apple (165g)			
Apricot	1 apricot (40g)			
Blackberries	1/3 cup (65g)			
Blueberries	¼ cup (40g)			
Blueberries, dried	1 Tbs (15g)			
Cherries, dried	1 Tbs (15g)			
Cherries, canned.	1 Tbs (20g)			
Cherries, raw	15 cherries (100g)			
Cranberries, dried	10 fruit (15g)			
Grapes (red or green)	10 grapes (50g)			
Jackfruit	¼ fruit (200g)			
Kumquats	2 fruit (40g)			
Nectarines	1 nectarine (160g)			

Orange, fruit	1 medium (200g)			
Orange juice, (fresh or concentrate)	1 cup or 250ml (260g)			
Paw paw	½ medium (100g)			
Peach, dried	¼ cup (40g)			
Peach, yellow	1 peach (180g)			
Pear	1 medium (185g)			
Pear, dried	1/3 cup (65g)			
Persimmons	1 fruit (75g)			
Plum, non-native	1 plum (70g)			
Raspberries	¼ cup (35g)			
Raspberries, frozen	¼ cup (35g)			
Strawberries	4 berries (75g)			
Vegetables:				
Artichoke	½ whole (65g)			
Asian greens, e.g. bok choy	1 cup (130g)			
Asparagus, cooked	3 spears (35g)			
Avocado	¼ avocado (40g)			
Beans, snap	5 beans (20g)			
Broccoli, cooked	4 florets (80g)			
Brussels sprouts	3 sprouts (65g)			
Cabbage, red, raw	½ cup (50g)			
Capsicum (any colour)	¼ whole (70g)			
Carrot, orange, cooked	1 medium (115g)			
Carrot, orange, raw	1 medium (125g)			
Celery	4 sticks (30g)			
Corn, sweet, yellow	½ medium cob (80g) OR ¼ cup kernels (90g)			
Cress, garden	1 cup (35g)			

Edamame	½ cup (95g)			
Fennel bulb	½ cup (75g)			
Kale	1 cup (115g)			
Kale, cooked	½ cup (60g)			
Leek, cooked	¼ cup (25g)			
Lettuce, cos or romaine	1 cup (35g)			
Okra	1 okra (10g)			
Okra, cooked	1 okra (10g)			
Olives, canned/jar	4 whole (15g)			
Pea, green, cooked	¼ cup (40g)			
Peppers, jalapeno	1 (20g)			
Pumpkin, cooked	2 medium pieces (190g)			
Rocket	½ cup (20 g)			
Rhubarb	½ stalk (75g)			
Sauerkraut	¼ cup (50g)			
Silverbeet, cooked	¾ cup (85 g)			
Snacks, popcorn, air-popped	1 cup (7g)			
Spinach, baby	1 cup (45g)			
Spinach, baby, raw, cooked	1/3 cup (40g)			
Spinach, baby, frozen, cooked	1/3 cup (40g)			
Tomatoes, canned	¼ cup (60g)			
Tomatoes, sun-dried	3 slices (15g)			
Watercress	¾ cup (25g)			
Zucchini	½ zucchini (100g)			
Zucchini, cooked	½ zucchini (85g)			
Grains:				
Barley, pearled, cooked	½ cup (95g)			

Bread (all types)	1 slice (40g)			
Oats	½ cup oats (uncooked) (40g)			
Pasta (wholemeal or white)	½ cup (75g)			
Rice (all types)	½ cup (100g)			
Quinoa, raw	1/3 cup (60g)			
Lean meat and poultry, fish, eggs, tofu, nuts and seeds and legumes/beans				
Beans and legumes (all types, e.g. kidney beans, lentils)	1 cup (150g)			
Egg, whole, cooked	2 (80g)			
Flaxseeds	½ Tbs (7g)			
Fish, fillet, cooked	1 fillet 100 g			
Nuts, almonds, whole	2 Tbs (30g)			
Nuts, hazelnuts, whole	2 Tbs (20g)			
Nuts, peanuts, shelled	2.5 Tbs (30g)			
Nuts, pistachios, shelled	¼ cup (30g)			
Pepitas (seeds)	1 Tbs (10g)			
Poultry, cooked	80g (e.g half breast, 1 leg)			
Read meat, cooked (all types, e.g. beef, pork, lamb)	65g (e.g. small steak, ½ cup mince)			
Tofu	3 large cubes (170g)			
Milk, yoghurt, cheese and / or their alternatives				
Milk (all types)	1 cup or 250mL			
Cheese, hard (e.g. cheddar)	2 slices (40g)			
Yoghurt	¾ cup (170g)			
Other				
Biscuits, sweet	2-3 biscuits (30g)			
Cake or muffin	1 slice or muffin (40g)			

Chocolate	4 squares or 1 small bar (30g)			
Hot chips, fried	12 chips (60g)			
Ice cream	2 scoops (75 g)			
Lollies	5-6 small (40g)			

1.2 Please indicate below any supplements you are currently taking, or have taken in the last 7 days. (e.g. multivitamin, iron supplement)

Appendix B-3

Table 9-1 Tertile misclassification between monthly screeners, combined weekly screeners and 24-hour diet recalls

Cohort	Tool	Comparative tool	Percentage misclassification
Australia	MS1	MS2	5% upper adjacent tertile 24% lower adjacent tertile 2% opposite lower tertile
	MS2	CWS	29% upper adjacent tertile 9% lower adjacent tertile
	24DR	MS2	25% upper adjacent tertile 29% lower adjacent tertile 8% opposite tertile
	24DR	CWS	11% upper adjacent tertile 14% lower adjacent tertile 11% upper opposite tertile
UK	24DR	CWS	39% upper adjacent tertile 7% lower adjacent tertile

Abbreviations: MS1 monthly screener one, MS2 monthly screener two, 24DR 24-hour diet recall, CWS combined weekly screeners, UK United Kingdom

Appendix C

Appendix C-1: Electronic Device Use Questionnaire

Electronic Device Use Questionnaire (EDUQ):

(Derived from Williams et al. 2019[1])

The following questionnaire covers questions regarding general health, work history, education and physical acitivty behaviours. It also explores behaviours and habits surrounding electronic device use

Date: Name:

DOB:

Sex: Female Male Other, please specify:

Country of Residence: Post-code of Residence:

1. **Medical History:**

1.1 Weight (kg):

1.2 Height (cm):

1.3 Do you have any chronic health conditions? (e.g. High blood pressure) Yes No

If yes please specify:

1.4 Do you have any family history of a condition known as age-related macular degeneration? Yes No

If yes, please specify their relationship to you (e.g. mother):

1.5 Do you have any family history of glaucoma? Yes No

If yes, please specify their relationship to you (e.g. mother):

1.6 Do you have any family history of retinitis pigmentosa? Yes No

If yes, please specify their relationship to you (e.g. mother):

1.7 Have you ever taken or are you currently taking a supplement that contains Lutein and/or Zeaxanthin and/or Meso-zeaxanthin? Yes No

Examples of common lutein/zeaxanthin/mesozeaxanthin supplements are: Blackmores Lutein Defence, Blackmores Lutein Vision-Advanced, Blackmores MacuVision, Healthy Care Bilberry and Lutein, Wagner Bilberry and Lutein, Australian Natural Care Healthy Eyes, Ocuvite Lutein.

Note: Multi-vitamins do not usually contain lutein/zeaxanthin/mesozeaxanthin. However, Swisse Ultivite contains lutein, if you take this please indicate this.

If yes, please specify the name of the supplement, when you have been taking the supplement and for how long:

1.8 Please list any other medications or supplements you are currently taking:

_____

_____

2. Education and Occupation:

2.1. What highest level of education have you **completed**? (please tick)

___ Grade 10 School Completion

___ Grade 12 School Completion

___ TAFE certificate

___ TAFE diploma

___ Trade apprenticeship certificate (e.g. carpentry)

___ Undergraduate University Degree

___ Masters University Degree

___ PhD

2.2 What is your **current** occupational status? (please tick)

___ Student

___ Employed / Self-Employed

___ On Leave (e.g. Maternity)

___ Unemployed

___ Retired

2.3 Work history:

Please fill out the table below to provide information about your current and past professional occupations, (inclusive of casual/part-time/permanent).

Only fill as many as needed or up to 20 years ago.

Job Title (e.g. receptionist, coach, plumber, psychologist, sales assistant)	Type of work (e.g. admin, labourer, health, marketing, politics)	Number of months / years in role	I work outdoors over 50% of time in this role (Yes/No)	I look at electronic device screens over 50% of time in this role (Yes/No)

3 Electronic Device Use:

The following questions are about how you use electronic devices each day and how this has changed over your lifetime. For the purpose of this survey electronic devices include the following:

- Smartphones e.g. iPhone, Samsung, Huawei.
- Computers/laptops e.g. Dell, Microsoft, MacBook.
- Tablets e.g. Surface Pro, iPad.
- Television/Projector Screen e.g. TV, movie theatres, lecture/conference halls screens, meeting room screens.

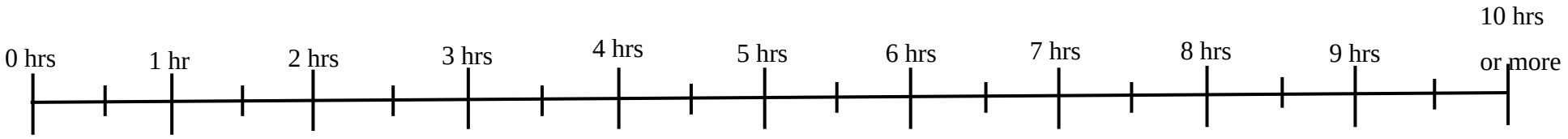
Note: For the purpose of this survey, using and electronic device is when you are looking at it and using it. For example, having the TV on in the background, but you are not actually looking directly at it does not count toward time using electronic devices.

3.1 Thinking back over the last 3 months, please indicate the number of hours you normally spend performing the following activities on an average day.

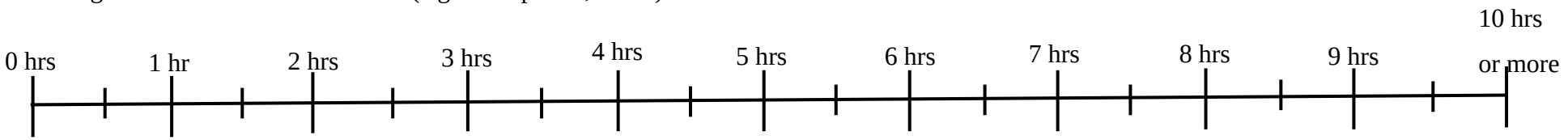
Weekday (i.e. Monday-Friday):

Viewing a TV screen (e.g. movies, video games, news)

Viewing a computer screen (e.g. laptop, desktop, computer games)

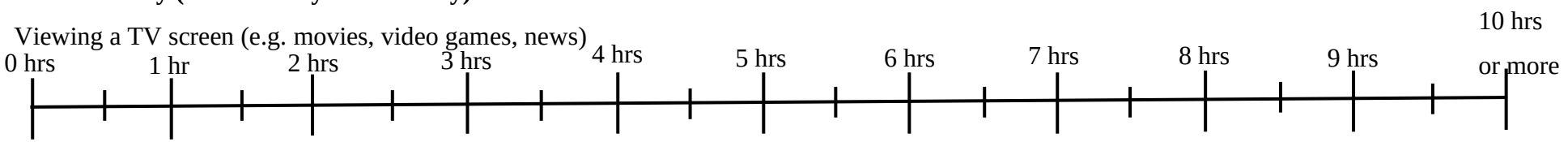


Viewing a handheld electronic device (e.g. smartphone, tablet)

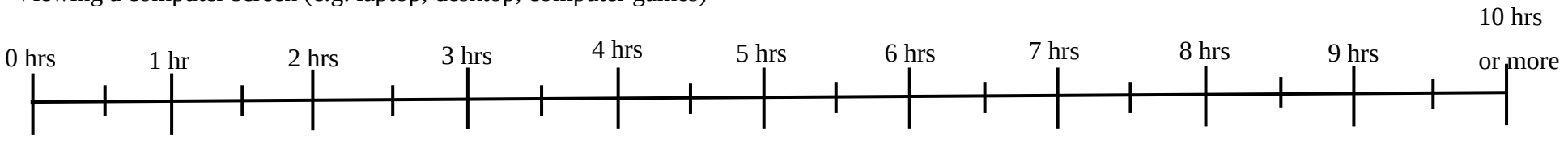


Weekend Day (i.e. Saturday and Sunday):

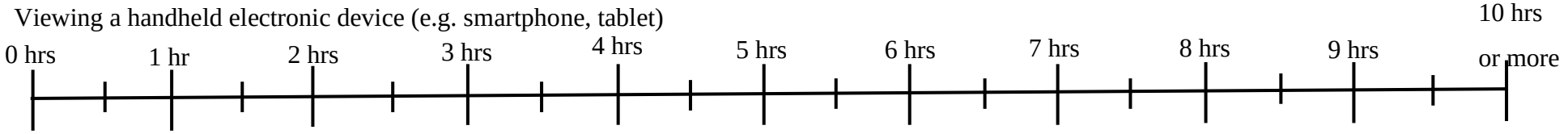
Viewing a TV screen (e.g. movies, video games, news)



Viewing a computer screen (e.g. laptop, desktop, computer games)



Viewing a handheld electronic device (e.g. smartphone, tablet)



Adapted from Williams et al. (2019)[1]

3.2 For each time point below please circle whether your daily use of electronic devices has either increased, decreased or remained the same:

If your daily use of electronic device has **<u>not changed</u>** at all over the last 20 years, why do you think this may be?

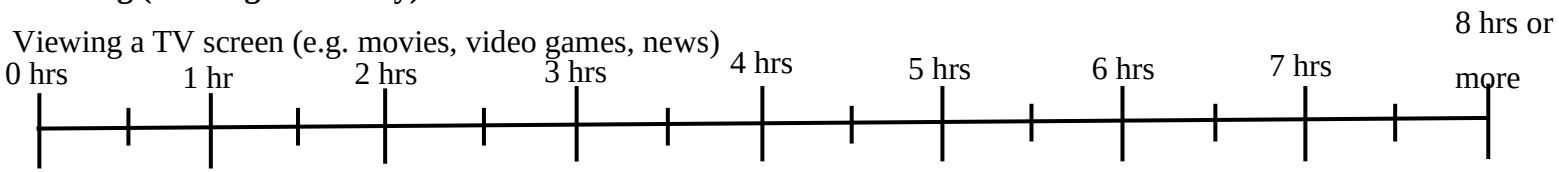
*Compared to **1** year ago my electronic device use has….* Increased Decreased Not Changed	If it has **increased or decreased** please specify the main reason you think this may be:
*Compared to **5** years ago my electronic device use has…* Increased Decreased Not Changed	If it has **increased or decreased** please specify the main reason you think this may be:
*Compared to **10** years ago my electronic device use has…* Increased Decreased Not Changed	If it has **increased or decreased** please specify the main reason you think this may be:
*Compared to **15** years ago my electronic device use has…* Increased Decreased Not Changed	If it has **increased or decreased** please specify the main reason you think this may be:
*Compared to **20** years ago my electronic device use has…* Increased Decreased Not Changed	If it has **increased or decreased** please specify the main reason you think this may be:

3.3 24 Hour Recall

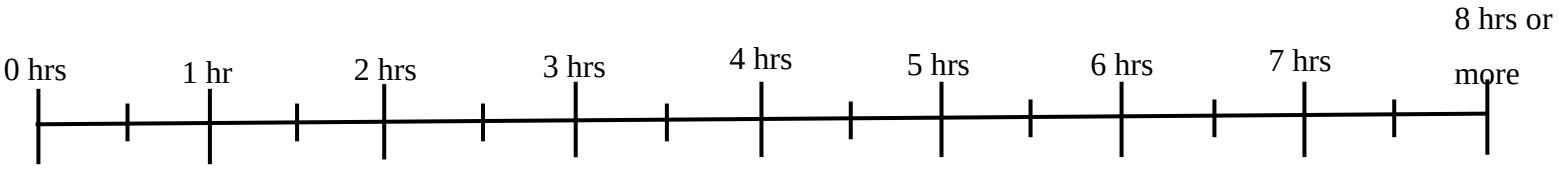
Thinking back to yesterday, please indicate how many hours you used the following electronic devices for the time brackets below (Morning, Afternoon, Evening):

Morning (Waking to Midday)

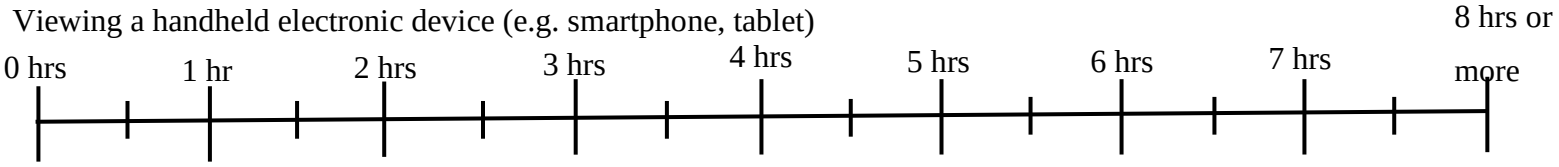
Viewing a TV screen (e.g. movies, video games, news)



Viewing a computer screen (e.g. laptop, desktop, computer games)

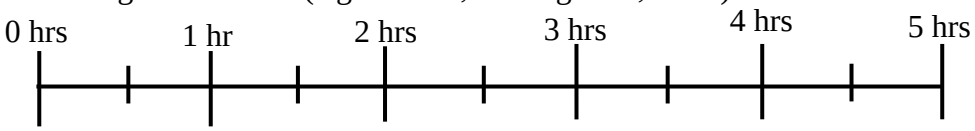


Viewing a handheld electronic device (e.g. smartphone, tablet)



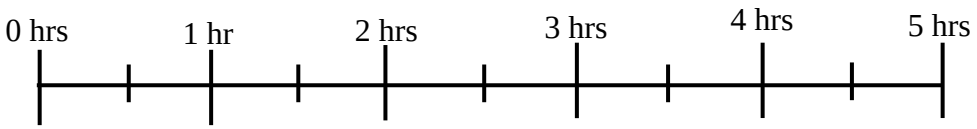
Afternoon (Midday to 5pm)

Viewing a TV screen (e.g. movies, video games, news)

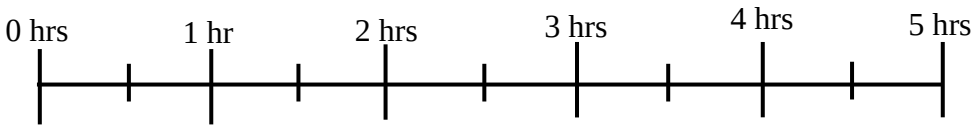


Question continues over page…

Viewing a computer screen (e.g. laptop, desktop, computer games)

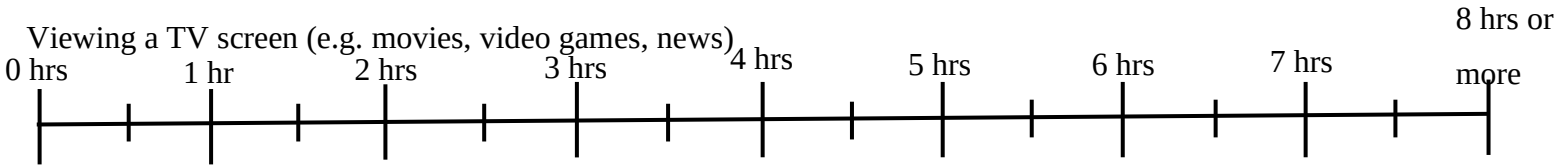


Viewing a handheld electronic device (e.g. smartphone, tablet)

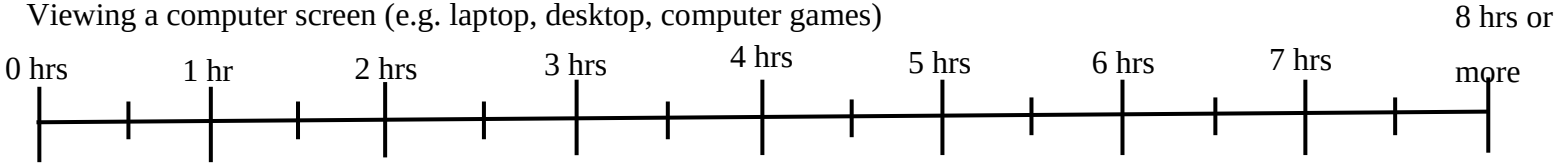


Evening (5pm to Sleep)

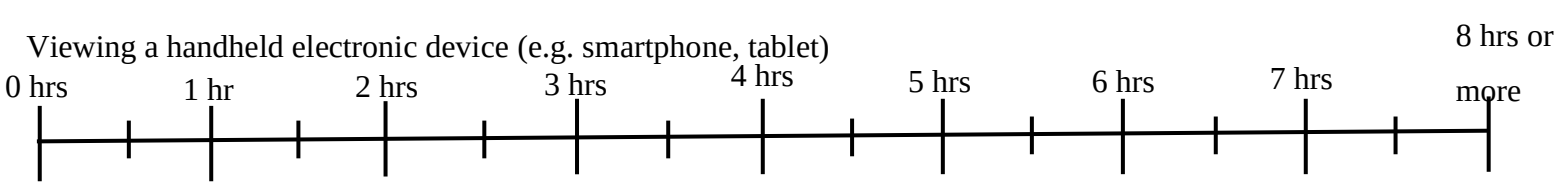
Viewing a TV screen (e.g. movies, video games, news)



Viewing a computer screen (e.g. laptop, desktop, computer games)



Viewing a handheld electronic device (e.g. smartphone, tablet)



3.4.1 Device reported screen time: If available, and turned on for your devices please indicate the screen time reports recorded by your device. *(Apple devices found in 'Settings' under 'Screen Time', Google devices found under 'Digital Wellbeing', other Android devices may require you to have downloaded an application that tracks screen time)*

Please include information such as daily average, number of times you picked up or opened the device, and average time spent using different types of applications e.g. games, social networking, other.

3.4.2 Did you use your devices screen time application to answer the previous questions? Yes No

If yes, please specify which questions below:

3.5 Settings of electronic devices you use.

3.5.1 Do you utilise settings on your electronic devices that change the colour of the screen to be more yellow?

On computers/laptops, tablets and smartphones there is often a setting available to change the colour of the screen to be more yellow (reduce blue light). It is usually aligned with your local sunset (turns more yellow) and sunrise (turns more blue).

Yes No

If yes, please specify below on what devices and when you use this setting.

3.6.1 Do you wear glasses (for visual correction, reading etc.)? Yes No

Note: Even if you only wear glasses for certain activities please circle yes.

3.6.2 If yes, when using electronic devices please circle how much of the time do you wear your glasses?

Never

Rarely

Some of the time

Most of the time

Always

3.6.3 Does the lens of your glasses filter a portion of blue light entering your eye? Yes No Unsure

If yes, please specify below how much if known (e.g. filters 25% OR provide brand name of technology e.g. Baxter Blue).

4 Physical Activity and Sleep

Note: For the purpose of this survey physical activity includes things such as walking, swimming, gardening, hiking, organised sport (e.g. netball, soccer) etc.

4.1 How many times per week do you participate in leisure-time physical activity? _____

4.2 How many hours per week do you participate in leisure-time physical activity? _____

4.3 When outdoors do you were sunglasses… (please circle)

Never

Rarely

Some of the time

Most of the time

Always

4.4 How many hours of sleep do you normally get on a week night? _____

Weekend night? _____

Thank you for completing this survey!

References:

1. Williams R, Bakshi S, Ostrin EJ, Ostrin LA. Continuous Objective Assessment of Near Work. Scientific reports. 2019;9(1):6901.

Appendix C-2: 24-hour electronic device use diary

Time			Minutes Electronic Device Used		
			Viewing a TV screen (e.g. movies, video games, news)	Viewing a computer screen (e.g. laptop, desktop, computer games)	Viewing a handheld electronic device (e.g. smartphone, tablet)
12:00:00 AM	to	12:14:00 AM			
12:15:00 AM	to	12:29:00 AM			
12:45:00 AM	to	12:59:00 AM			
1:15:00 AM	to	1:29:00 AM			
1:45:00 AM	to	1:59:00 AM			
2:15:00 AM	to	2:29:00 AM			
2:45:00 AM	to	2:59:00 AM			
3:15:00 AM	to	3:29:00 AM			
3:45:00 AM	to	3:59:00 AM			
4:15:00 AM	to	4:29:00 AM			
4:45:00 AM	to	4:59:00 AM			
5:15:00 AM	to	5:29:00 AM			
5:45:00 AM	to	5:59:00 AM			
6:15:00 AM	to	6:29:00 AM			
6:45:00 AM	to	6:59:00 AM			
7:15:00 AM	to	7:29:00 AM			
7:45:00 AM	to	7:59:00 AM			
8:15:00 AM	to	8:29:00 AM			
8:45:00 AM	to	8:59:00 AM			
9:15:00 AM	to	9:29:00 AM			
9:45:00 AM	to	9:59:00 AM			
10:15:00 AM	to	10:29:00 AM			
10:45:00 AM	to	10:59:00 AM			
11:15:00 AM	to	11:29:00 AM			

11:45:00 AM	to	11:59:00 AM			
12:15:00 PM	to	12:29:00 PM			
12:45:00 PM	to	12:59:00 PM			
1:15:00 PM	to	1:29:00 PM			
1:45:00 PM	to	1:59:00 PM			
2:15:00 PM	to	2:29:00 PM			
2:45:00 PM	to	2:59:00 PM			
3:15:00 PM	to	3:29:00 PM			
3:45:00 PM	to	3:59:00 PM			
4:15:00 PM	to	4:29:00 PM			
4:45:00 PM	to	4:59:00 PM			
5:15:00 PM	to	5:29:00 PM			
5:45:00 PM	to	5:59:00 PM			
6:15:00 PM	to	6:29:00 PM			
6:45:00 PM	to	6:59:00 PM			
7:15:00 PM	to	7:29:00 PM			
7:45:00 PM	to	7:59:00 PM			
8:15:00 PM	to	8:29:00 PM			
8:45:00 PM	to	8:59:00 PM			
9:15:00 PM	to	9:29:00 PM			
9:45:00 PM	to	9:59:00 PM			
10:15:00 PM	to	10:29:00 PM			
10:45:00 PM	to	10:59:00 PM			
11:15:00 PM	to	11:29:00 PM			
11:45:00 PM	to	11:59:00 PM			

5715 Would you say this is a typical day of device use for you? Yes No *If no, please indicate why.*

Appendix C-3

Table 9-2 Calculations for determining daily hours of electronic device use

Tool	Electronic device category	Equation
EDUQ	All devices	Mean daily hours of use from all devices combined = ((weekday TV+ weekday computer + weekday handheld) × 5 + (weekend TV + weekend computer + weekend handheld) × 2) ÷7
	Television	Mean daily hours of use from TV= ((weekday TV × 5) + (weekend TV × 2) ÷ 7
	Computer	Mean daily hours of use from computer = ((weekday computer × 5) + (weekend computer × 2) ÷ 7
	Handheld	Mean daily hours of use from handheld = ((weekday handheld × 5) +(weekend handheld × 2) ÷ 7
24-hour electronic device use diary	All devices	Mean daily hours of use from all devices combined = (sum of use from all completed diaries for TV + computer + handheld) ÷ number of diaries completed
	Television	Mean daily hours of use from TV = (sum of use from all completed diaries for TV) ÷ number of diaries completed
	Computer	Mean daily hours of use from computer = (sum of use from all completed diaries for computer) ÷ number of diaries completed
	Handheld	Mean daily hours of use from handheld = (sum of use from all completed diaries for handheld) ÷ number of diaries completed

Abbreviations: EDUQ, electronic device use questionnaire

Appendix C-4

Table 9-3 Change in device use over last 1–20 years as per EDUQ 1

Aus n = 56 UK n = 24		1 year ago	5 years ago	10 years ago	15 years ago	20 years ago
Participants reporting increase (%)	Aus	32	72	88	93	91
	UK	50	88	92	96	100
Participants reporting decrease (%)	Aus	5	2	4	2	2
	UK	8	0	0	0	0
Participants reporting no change (%)	Aus	63	26	9	5	7
	UK	42	13	8	4	0

Abbreviations: EDUQ1, Electronic Device use Questionnaire from week 1; Aus, Australia; UK, United Kingdom; n =, number of participants.

Appendix D

Appendix D-1

Table 9-4 Dietary intake of lutein and zeaxanthin

		Median (25th – 75th percentile)	Range
24-Hour Diet Recall: Reported L/Z intake (mg)		1.9 (0.9 – 4.9)	0.1 – 16.2 mg
Dietary L/Z screener: Total L/Z intake over month		129 (76 –208) mg	11 – 626 mg
Dietary L/Z Screener: Mean daily L/Z intake		4.6 (2.7 – 7.4) mg/day	0.4 – 22.35 mg/day
Dietary L/Z Screener: L/Z intake from each food group as a percentage of total L/Z intake over the month.	Fruit	3 (1.3 – 5.7) %	
	Vegetables	91 (83.9 – 93.9) %	
	Grains	1 (0.8 – 2.1) %	
	Milk, yoghurt, cheese, and alternatives	0.3 (0.1 – 0.5) %	
	Meat and meat alternatives	3 (1.4 – 6.4) %	
	Discretionary foods	0.2 (0.1 – 0.4) %	

Abbreviations: L/Z, lutein and zeaxanthin; %, percent; mg, milligrams.

Appendix D-2

Table 9-5 Foods with high contribution to total lutein and zeaxanthin intake from the monthly screener

Food	Baby spinach	Broccoli	Lettuce, Cos or Romaine	Orange carrot	Pumpkin	Zucchini
%	14.5 (1.2 – 26.3)	5.0 (0.0 – 10.2)	4.6 (1.1 – 8.6)	3.0 (0.0 – 9.0)	2.9 (0.0 – 8.5)	2.8 (0.0 – 5.8)

Baby spinach, broccoli, and orange carrot were raw, all other foods cooked.

Appendix E

Appendix E-1: Broccoli

Table 9-6 Broccoli, comparison of method variations 1 and 2

Sample ID	Lutein or zeaxanthin	Method variation (mean ± SD μg/100g) *	
		1	2
2A (n 3)	Lutein	886 ± 125	1,080 ± 95
	Zeaxanthin	BDL	BDL
2B (n 3)	Lutein	696 ± 115	1,078 ± 230
	Zeaxanthin	BDL	BDL
2C (n 2)	Lutein	537 ± 12	873 ± 149
	Zeaxanthin	BDL	BDL
2D (n 4)	Lutein	442 ± 209	670 ± 124
	Zeaxanthin	28 ± 3.6	33 ± 5.3
2E (n 4)	Lutein	276 ± 39	240 ± 26
	Zeaxanthin	BDL	BDL
2F (n 4)	Lutein	772 ± 100	772 ± 104
	Zeaxanthin	BDL	BDL

* Paired, two-tailed t-test comparing variation 1 and 2 for lutein, all samples together (Including Sample 2G and 2H), p = 0.12. Abbreviations: BDL, below detection limit; SD, standard deviation; n, number of replicates analysed per sample.

Table 9-7 Broccoli, comparison of method variations 1, 2, 3, and 4 with Sample 2 G and 2H

Sample ID	Method variation	Mean ± SD lutein (μg/100g)	Mean ± SD zeaxanthin (μg/100g)
2G (n 2)	1	1150 ± 95	BDL
	2	929 ± 65	BDL
	3	806 ± 90 [a]	BDL
	4	962 ± 67	BDL
2H (n 3)	1	729 ± 82	BDL
	2	855 ± 60	BDL
	3	675 ± 40 [a]	BDL
	4	1,040 ± 100	BDL

Kruskal-Wallis test and Dunn's multiple comparisons comparing between method variations of data pooled from 2G and 2H. [a] Method variation significantly different to variation 4 p = 0.02. Abbreviations: SD, standard deviation; ID, identification letter for sample; BDL, below detection limit; n, number of replicates analysed per sample

Table 9-8 Broccoli, comparison of method variations 5, 9, and 10 with. Sample 2I

Sample ID	Method variation	Mean ± SD lutein (μg/100g)	Mean ± SD zeaxanthin (μg/100g)
2I (n 7) *	5	613 ± 130	BDL
	9	633 ± 75	BDL
	10	587 ± 37 [a, b]	BDL

* No significant differences present between method variations. [a] Percentage assay return significantly different to variation 5 p = 0.04. [b] Percentage assay return significantly different to

variation 9 p = 0.007. Abbreviations: SD, standard deviation; ID, identification letter for sample; BDL, below detection limit; n, number of replicates analysed per sample

For Broccoli, of variations 1 to 4, variation 4 was significantly more optimal than variations 3 but not any different to variations 1, 2 (Table 9-7). Of variations 5, 9, 10 there were no significant differences between the variations. Mean percentage assay recovery measured with Sample 2G: variation 1 = 40.4%, variation 2 = 74%, variation 3 = 42.0%, variation 4 = 42.2%. No significant differences were present between percentage method recovery for variations 1 to 4. Mean percentage method recovery measured with Sample 2I: variation 5 = 78.5%, variation 9 = 87.3%, variation 10 = 60.2%. Variation 10 percentage recovery was significantly lower than variations 5 and 9 (p = 0.04 and p=0.007 respectively).

Appendix E-2: Broccolini

Table 9-9 Broccolini, comparison of method variations 1 and 2 with Sample 3A, and method variations 1 to 4 with Sample 3B

Sample ID	Method variation	Mean ± SD lutein (μg/100g)	Mean ± SD zeaxanthin (μg/100g)
3A (n 4)	1	3,121 ± 144	50 ± 13
	2	2,462 ± 384 [a]	81 ± 21 [a]
3B (n 3)	1	1,795 ± 95	32 ± 3.5
	2	2,114 ± 9 [a]	41 ± 0.9
	3	1,927 ± 32	33 ± 6.5
	4	2,074 ± 51	41 ± 3.2

Sample 3A, unpaired two-tailed t-test (L p = 0.0182, Z p = 0.0409). Sample 3B, Kruskal-Wallis test and Dunn's multiple comparison, L summary p = 0.0006, Z p = 0.012. [a] method variation significantly different to variation 1. Abbreviations: SD, standard deviation; ID, identification letter for sample; L, lutein; Z, zeaxanthin; n, number of replicates analyzed per sample

Table 9-10 Broccolini, comparison of method variations 5, 7, 9, and 10 with Sample 3C

Sample ID	Method variation	Mean ± SD lutein (μ/100g)	Mean ± SD zeaxanthin (μg/100g)
3C (n 7 ^)	5	1,677 ± 220	32 ± 3.9
	7	1,499 ± 74	30 ± 5.3 [a]
	9	2,386 ± 73 [a, b]	41 ± 2.2 [a, b]
	10	1,907 ± 361 [b, c]	46 ± 4.5 [a, b]

^ 7 replicates per method variation except variation 7, only 5 replicates (two lost in analysis). One-way ANOVA and Tukey's multiple comparisons. ANOVA summary L and Z p <0.0001. [a], method variation significantly different to variation 5; [b], method variation significantly different to variation 7; [c], method variation significantly different to variation 9. Abbreviations: SD, standard deviation; ID, identification letter for sample; n, number of replicates analysed per sample

For broccolini using variations 1 and 2, variation 1 was more optimal for L ($p = 0.0182$) but variation 2 more optimal for Z ($p = 0.0409$) (Table 9-9). Of method variations 1 to 4, variation 2 was most optimal (Table 11-9). Variation 1 was significantly less optimal than variation 2 ($p = 0.028$). No other significant comparisons were present. Overall, variation 9 was optimal for L (Table 9-10). Variations 5, 7, and 10 were significantly less optimal than variation 9 ($p < 0.0001$, $p < 0.0001$, and $p = 0.0035$ respectively). Variation 7 was significantly less optimal than variation 10 ($p = 0.0263$). Variations 9 or 10 were optimal for Z, but the method percentage recovery was higher for variation 9. For Z, variation 5 was significantly less optimal than variations 9 and 10 ($p = 0.0026$, and $p < 0.0001$ respectively), but more optimal than variation 7 ($p < 0.0001$). Variation 7 was significantly less optimal than variations 9 and 10 ($p < 0.0001$). The mean percentage method recovery: variation 5 = 24%, variation 7 = 21%, variation 9 = 88%, variation 10 = 55%. Variation 9 method recovery was significantly greater than all other variations ($p < 0.005$).

Appendix E-3: Baby orange capsicum

Table 9-11 Baby orange capsicum, comparison of method variations 1 and 2 with Sample 4A, method variations 1 and 3 with Sample 4B, and method variations 1 to 4 with Sample 4C

Sample ID	Method variation	Mean ± SD lutein (μg/100g)	Mean ± SD zeaxanthin (μg/100g)
4A (n 4)	1	170 ± 16	167 ± 15
	2	139 ± 20 [a]	129 ± 17 [a]
4B (n 4)	1	854 ± 20	1,031 ± 19
	3	886 ± 8 [a]	1,039 ± 11
4C (n 3)	1	516 ± 43	872 ± 74
	2	481 ± 29	828 ± 37
	3	513 ± 4	884 ± 16
	4	411 ± 118	712 ± 209

[a] Method variation significantly different to variation 1. Sample 4A, unpaired two-tailed t-test (L $p = 0.048$, Z $p = 0.015$). Sample 4B, L unpaired two-tailed t-test ($p = 0.023$), Z Mann-Whitney two-tailed test, no significant differences ($p = 0.34$). Sample 4C, L Kruskal-Wallis test and Dunn's multiple comparisons L $p = 0.10$, Z $p = 0.22$. Abbreviations: SD, standard deviation; ID, identification letter for sample; n, number of replicates analysed per sample

Table 9-12 Baby orange capsicum, comparison of method variations 5, 8, 9, and 10 with Sample 4D

Sample ID	Method variation	Mean ± SD lutein (μg/100g)	Mean ± SD zeaxanthin (μg/100g)
4D (n 7)	5	883 ± 18	1,592 ± 62
	8	862 ± 65	1,551 ± 117
	9	1,384 ± 84 [a, b]	2,948 ± 156 [a, b]
	10	1,022 ± 201 [c]	1,482 ± 170

L one-way ANOVA and Tukey's multiple comparisons $p < 0.0001$. Z Kruskal-Wallis test and Dunn's multiple comparisons $p = 0.001$. [a], method variation significantly different to variation 5; [b],

method variation significantly different to variation 7; [c], method variation significantly different to variation 9. Abbreviations: SD, standard deviation; ID, identification letter for sample; n, number of replicates analysed per sample

For baby orange capsicum in Sample 4A, variation 1 compared with variation 2 indicated variation 1 was more optimal for L ($p = 0.048$), but variation 2 was optimal for Z ($p = 0.015$) (Table 9-11). Sample 4B variation 1 compared to variation 3, variation 3 was optimal for L ($p = 0.023$) and no significant differences for were present for Z. Sample 4C, no significant differences were present between variations for L ($p = 0.10$) or Z ($p = 0.22$). Sample 4D, variation 9 was optimal for L and variation 9 or 10 for Z. Variation 9 L concentrations were higher compared with variations 5, 8, and 10 (L $p < 0.0001$ for all). Variation 9 Z concentrations were higher compared with variations 5, 8, but not 10 ($p = 0.019$, $p = 0.001$, and $p = 0.18$ respectively) (Table 9-12). The mean percentage method recovery for variations 5, 8, 9, and 10 were not significantly different. Method recovery for each variation was: variation 5 = 92%, variation 8 = 86%, variation 9 = 84%, variation 10 = 84%.

Appendix E-4: Dried goji berries

Table 9-13 Dried goji berries, comparison of method variations 1, 2, 3, and 4 with Sample 5A

Sample ID	Method variation	Mean ± SD lutein (μg/100g)	Mean ± SD zeaxanthin (μg/100g)
5A (n, 4)	1	BDL	900 ± 99
	2	BDL	745 ± 43
	3	BDL	1,103 ± 69 [a]
	4	BDL	751 ± 50 [b]

Kruskal-Wallis test and Dunn's multiple comparisons Z $p = 0.0006$. [a], method variation significantly different to variation 2 ($p = 0.014$). [b], method variation significantly different to variation 3 ($p = 0.023$). Abbreviations: SD, standard deviation; ID, identification letter for sample; BDL, below detection limit; n, number of replicates analysed per sample

Table 9-14 Dried goji berries, comparison of method variations 5, 9, and 10 with Sample 5B

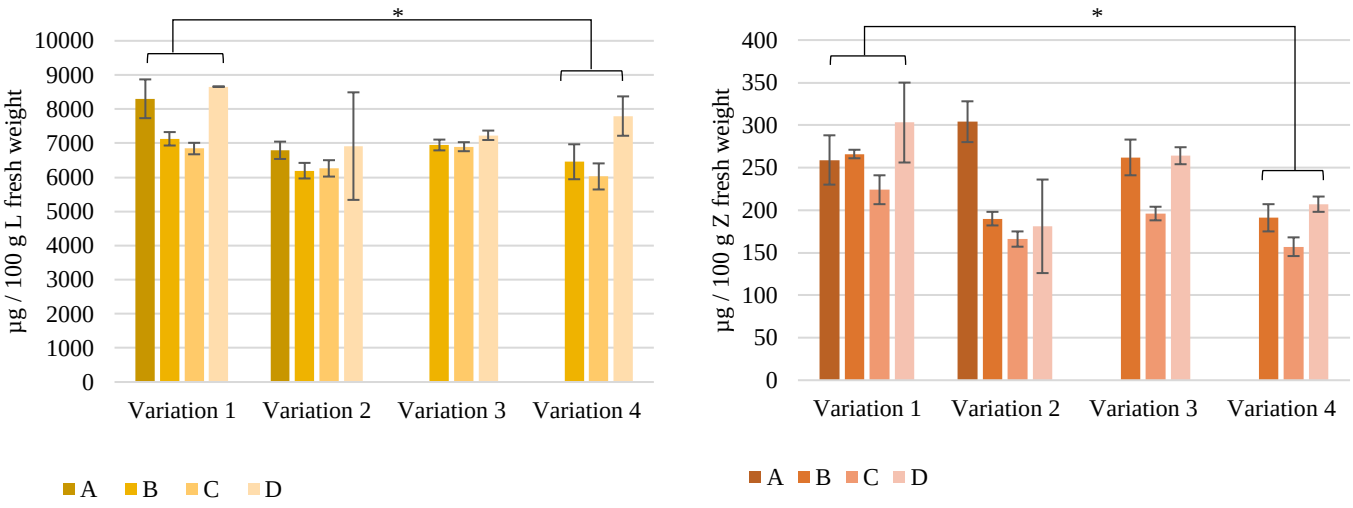
Sample ID	Method variation	Mean ± SD lutein (μg/100g)	Mean ± SD zeaxanthin (μg/100g)
5B (n = 7)	5	101 ± 5.3	826 ± 38
	9	231 ± 9.7 [a]	1,586 ± 126 [a]
	10	119 ± 9.1	687 ± 36 [b]

Kruskal-Wallis test and Dunn's multiple comparisons L and Z $p < 0.0001$. [a], method variation significantly different to variation 5; [b], method variation significantly different to variation 9. Abbreviations: SD, standard deviation; ID, identification letter for sample; n, number of replicates analysed per sample

In Sample 5A of the dried goji berries, of variations 1 to 4, variation 3 was significantly greater than variation 2 ($p = 0.014$) and 3 ($p = 0.023$) but no different to 1 (Table 9-13). L was below the detection limit. Sample B, variation 9 was most optimal for Z and L (Table 9-14). Variation 9 was significantly more optimal than variations 5 for L ($p = 0.001$) and 5 and 10 for Z ($p = 0.018$ and $p = 0.008$ respectively). The mean percentage method recovery for variations 5, 9, 10 were not significantly different. Mean percentage recovery: variation 5 = 69%, and variation 9 = 73%. The median percentage return for variation 10 was 33%.

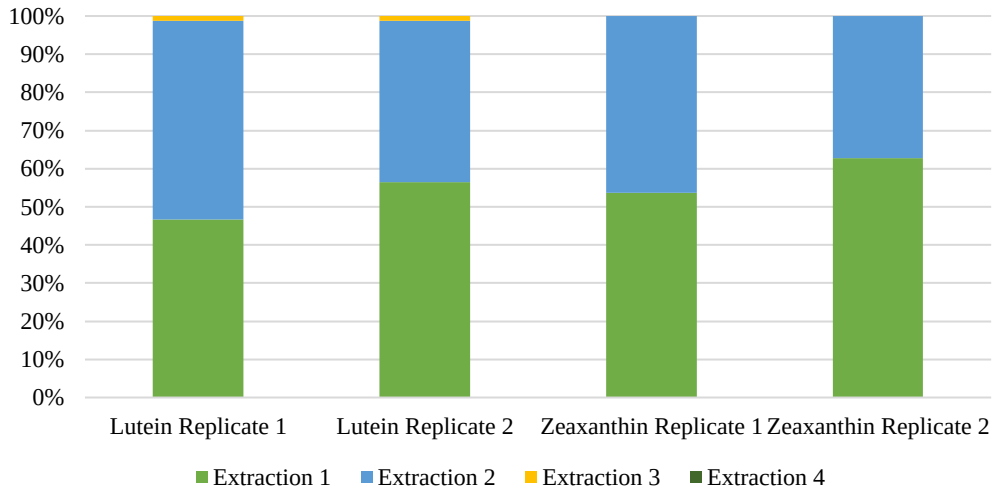
Appendix E-5: Baby spinach, graphical representation of outcomes

Graphical representation of Chapter 5 baby spinach extraction method variation outcomes.



(**a**) (**b**)

Figure 9-1 Baby spinach mean concentrations measured using variations 1 to 4
Three replicates per sample except Sample 1D (2 replicates), variation 3 and 4 not completed for



Sample 1A, error bars indicate standard deviation of mean: (**a**) Lutein concentrations for Samples 1A, 1B, 1C and 1D; (**b**) Zeaxanthin concentrations for samples 1A, 1B, 1C, and 1D. * $p \leq 0.01$

Figure 9-2 Baby spinach, Sample 1E, percentage of total lutein and zeaxanthin measured per extraction (four extractions total), measured with variation 5

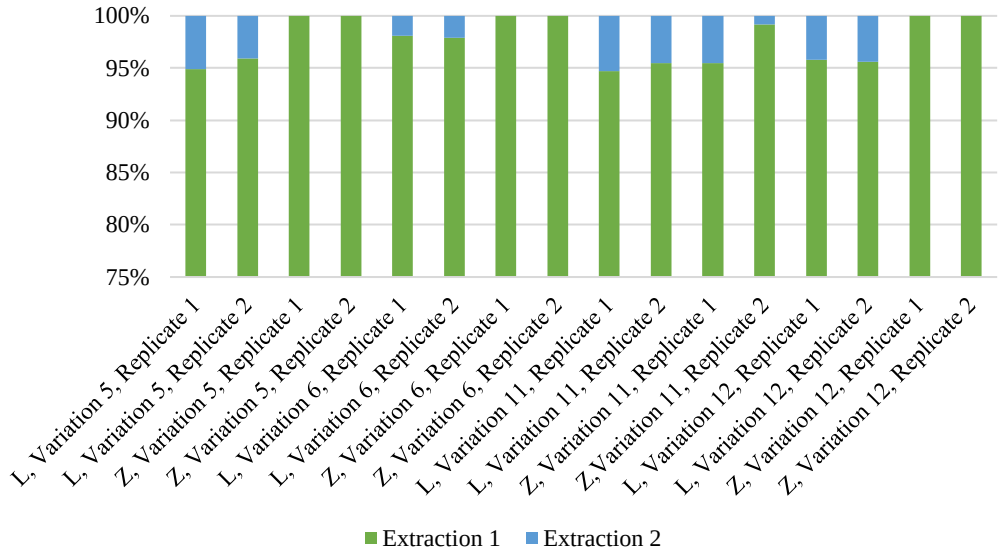


Figure 9-3 Baby spinach, Sample 1F, percentage of total lutein and zeaxanthin measured per extraction (two extractions total), measured with variations 5, 6, 11, and 12
Abbreviations: L, lutein; Z, zeaxanthin

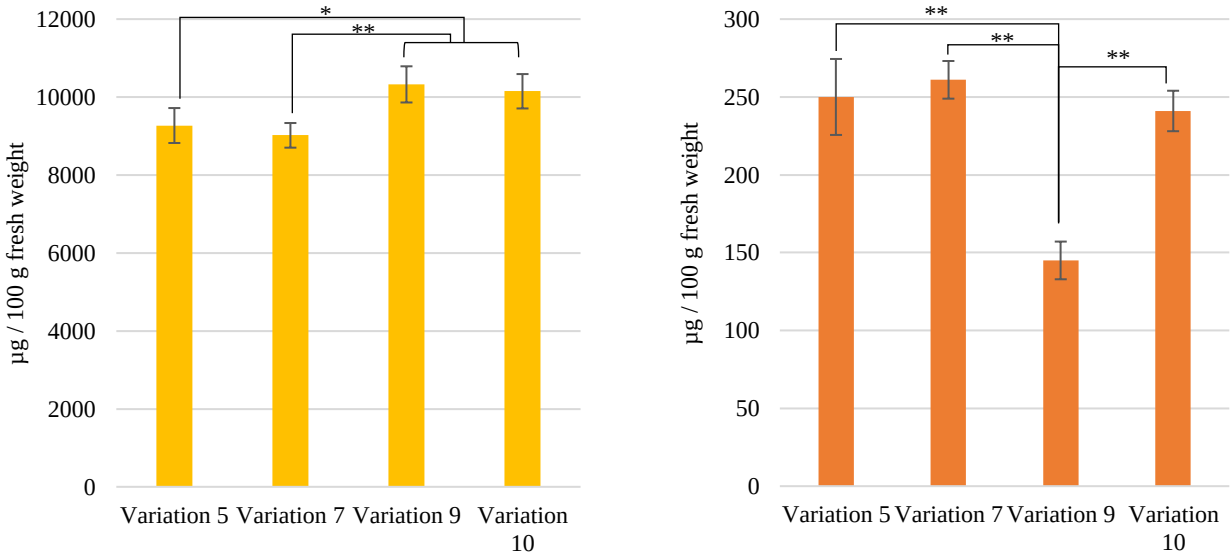


Figure 9-4 Baby spinach, Sample 1G, mean concentrations measured using variations 5, 7, 9, and 10 Seven replicates per sample, error bars indicate standard deviation of mean: (**a**) Lutein concentrations; (**b**) Zeaxanthin concentrations. * $p < 0.005$. ** $p < 0.0005$

Appendix E-6: Food sample growing and purchase location

Table 9-15 Food sample purchase and growing location information

Food type	Sample identification	Purchase Month, Year	Store of purchase, suburb of purchase in Queensland (postcode of suburb)	Product brand or supplier	Location product grown in
Broccoli	A	February, 2020	Woolworths, Coorparoo, 4151	Tofflon Bros Pty Ltd	Werribee South, VIC, Australia, 3030
	B	February, 2020	Aldi, Stones Corner, 4120	Raw Fresh Pty Ltd	Australia, further details unknown
	C	February, 2020	Rock n Roll Deli, Greenslopes, 4120	Harvest Moon Pty Ltd	Forth, TAS, Australia, 7310
	D	May, 2020	IGA Marketplace, Greenslopes, 4120	Unknown	Australia, further details unknown
	E	May, 2020	Aldi, Stones Corner, 4120	Rugby Farm Pty Ltd	Gatton, QLD, Australia 4343
	F	May, 2020	Woolworths, Coorparoo, 4151	Willow Springs Produce	Nobby, QLD, Australia, 4360
	G	August 2020	Milton Fruit Bowl, Milton, 4064	Unknown	Forrest Hill, QLD, Australia, 4342
	H	December, 2020	Fruity Capers and Deli, Toowong, 4066	Unknown	Australia, further details unknown
	I	May, 2021	Woolworths, Coorparoo, 4151	Unknown	Australia, further details unknown

Broccolini	A	May, 2020	Aldi, Stones Corner, 4120	Vanstone Produce Pty Ltd	Crowley Vale, QLD, Australia, 4342
	B	August, 2020	Fruity Capers and Deli, Toowong, 4066	Maragi Pty Ltd	Gatton, QLD, Australia, 4343
	C	July, 2021	Aldi, Stones Corner, 4120	Campsey Ash Farms Pty Ltd	Gatton, QLD, Australia, 4343
Baby orange capsicum	A	May, 2020	Aldi, Stones Corner, 4120	Kalfresh Pty Ltd	Kalbar, QLD, Australia, 4309
	B	June, 2020	Woolworths, Coorparoo, 4151	Perfection Fresh Australia Pty Ltd	Australia, further details unknown
	C	August, 2020	Aldi, Stones Corner, 4120	Kalfresh Pty Ltd	Kalbar, QLD, Australia, 4309
	D	April, 2021	Coles, Toowong, 4066	Coles	Australia, further details unknown
Goji Berries	A	June, 2020	Woolworths, Coorparoo, 4151	Woolworths	Product of China (packed in Australia)
	B	May, 2021	Woolworths, Coorparoo, 4151	Woolworths	Product of China (packed in Australia)
Spinach	A	May, 2020	Aldi, Stones Corner, 4120	The Fresh Salad Co.	Australia, further details unknown
	B	August, 2020	Aldi, Stones Corner, 4120	The Fresh Salad Co.	Australia, further details unknown

C	November, 2020	Woolworths, Coorparoo, 4151	Harvest Fresh Cuts Pty Ltd	Australia, further details unknown
D	December, 2020	Milton Fruit Bowl, Milton, 4064	Unknown	Australia, further details unknown
E	January, 2021	Rock n Roll Deli, Greenslopes, 4120	Unknown	Australia, further details unknown
F	January, 2021	IGA Marketplace, Greenslopes, 4120	Community and Co.	VIC, Australia, further details unknown
G	March, 2021	Woolworths, Woollongabba, 4120	Unknown	Australia, further details unknown

5858 Sample identification, each different sample of a food purchased is denoted by a different letter