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Original article

Building food composition tables: extraction methods to measure lutein and zeaxanthin concentrations in select Australian foods

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Summary The lutein and zeaxanthin (L/Z) in food composition tables has infrequently utilised methods optimised for L/Z and comprehensive data are absent, such as in Australia. These absences limit quality dietary intake research. This study investigated optimisation of extraction methods for lutein and zeaxanthin in five Australian foods analysed by high-performance liquid chromatography and photodiode array detection. The foods were broccoli, broccolini, baby spinach, baby orange capsicum, and dried goji berry. Twelve variations in extraction methods were investigated, including saponification, sonication, and solvent choice. L/Z concentrations differed by up to more than 125% between variations. Variation nine was best for all foods except zeaxanthin in broccoli where variation five or seven were best. The L/Z concentrations measured differed in Australian and United States data; existing data may therefore not be representative of the current food supply. Development of local Australian food composition data for lutein and zeaxanthin is warranted.

Keywords Food analysis, high-performance liquid chromatography, lutein, macular pigments, xanthophylls, zeaxanthin.

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) (Lupton *et al.*, 2014). 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 (Ma *et al.*, 2012). Many countries do not have comprehensive FCTs for lutein and zeaxanthin, one exception is the United States Department of Agriculture (USDA) tables (USDA, 2018). In countries without comprehensive tables, such as Australia, attempts to capture dietary lutein and zeaxanthin intake have relied upon the USDA tables (Tan *et al.*, 2008). The Food Standards Australia and New Zealand (FSANZ) FCTs are not comprehensive with only 26 entries for lutein (not zeaxanthin) (FSANZ, 2019a). 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% (USDA, 2018; FSANZ, 2019a).

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 (Britton *et al.*, 2009; Rodriguez-Amaya, 2010; Walsh *et al.*, 2015; Pintea *et al.*, 2020). 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

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(HPLC-DAD) (Rodriguez-Amaya, 2010; Pintea *et al.*, 2020). 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 (Rodriguez-Amaya, 2010; Rivera & Canela, 2012; Amorim-Carrilho *et al.*, 2014). 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 (Agarwal *et al.*, 2020). Therefore, optimising an extraction method to improve assay efficiency is important (Amorim-Carrilho *et al.*, 2014). 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 (Fanning *et al.*, 2010). For example, many of the entries in the USDA tables were not extracted and analysed using recent or lutein- and zeaxanthin-specific techniques (USDA, 2018). 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 (Government, 2022).

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 (Fitzpatrick *et al.*, 2022). 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.

Materials and methods

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.

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/100 g of lutein and zeaxanthin as per data from the USDA or FSANZ FCT (USDA, 2018; FSANZ, 2019a). Foods reported to contain more than 100 μg/100 g of lutein and zeaxanthin were selected to ensure high applicability to subsequent research on dietary lutein and zeaxanthin intake (Fitzpatrick *et al.*, 2022). 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 Data S5. The guideline document Generating Data for Food Standards Australia New Zealand Nutrient Databases (2019b) and the Food Composition Data book by Greenfield & Southgate (2003) were used to inform the sampling strategy and volume of food for purchase (Greenfield & Southgate, 2003; FSANZ, 2019b). 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 150 g, 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 1).

Table 1 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	1B = sample B of baby spinach
3 = broccolini	C = sample C	2A = sample A of broccoli
4 = baby orange capsicum	D = sample D	
5 = dried goji berry		

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

Food sample preparation

The shape and type of a food sample determined the preparation to obtain a 'reduced sample' (Greenfield & Southgate, 2003). 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 (Greenfield & Southgate, 2003). 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 1000 W 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 2.5 min for broccoli, and 2 min 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 handheld blender (Bamix® Mono blender 140 W). 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, 0.25 mL of distilled water per 1 g of food was added and blending attempted. If blending was still unsuccessful, the ratio of distilled water to reduced sample was increased in 0.05 mL increments until blending was successful. The volume of distilled water added per 1 g of food was 1 mL for broccoli, 1 mL for broccolini, 0.7 mL for baby spinach, and 1.5 mL for dried goji berry. Approximately 2 g of the blended food mixture was transferred to a 5 mL vial, and 2 mL 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 100–1000 µL pipette tip.

Lutein and zeaxanthin extraction

Analytical methods described by Chandra-Hioe *et al.* (2017) and Fanning *et al.* (2010) were used as the initial reference extraction methods. Briefly, 200 µL of prepared food sample and 400 µL of acetone was added to a 1.5 mL microfuge tube and mixed for 10 s. To the resulting solution, 600 µL of n-hexane was added, mixed for 10 s then centrifuged for 4 min at 12 000 r.p.m. (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 100 µL of mobile phase (methanol 49.96%, acetonitrile 49.96%, triethylamine 0.08%), mixed for 10 s 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 Fig. 1. Two variations occurred during the food sample preparation. The first was addition of 2 mL of ethanol instead of distilled water before homogenisation. The second was after homogenisation where the food sample was sonicated at 4 °C for 30 s (Qsonica Sonicators, Model CL-188). All other variations occurred after 200 µL 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 (Agarwal *et al.*, 2020), saponification of the sample, and two extractions of hexane or hexane/DCM rather than one. Saponification was achieved by addition of 150 µL of 10 N potassium hydroxide (KOH) and incubated in water at 45 °C for 30 min, or addition of 300 µL of methanol sodium hydroxide (MeOH NaOH) and incubated in water at 60 °C for 30 min.

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 5 µm RP-aqueous C30 140A, 250 × 4.6 mm column with isocratic mobile phase containing methanol (49.96%), acetonitrile (49.96%), and 0.08% triethylamine at a flow rate of 1.2 mL/min with a 30-min run time (Hart & Scott, 1995; Emenhiser *et al.*, 1996). Detection of lutein and zeaxanthin was performed at 445 nm (Wrolstad *et al.*, 2005; Fanning *et al.*, 2010).

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 eqn (1):

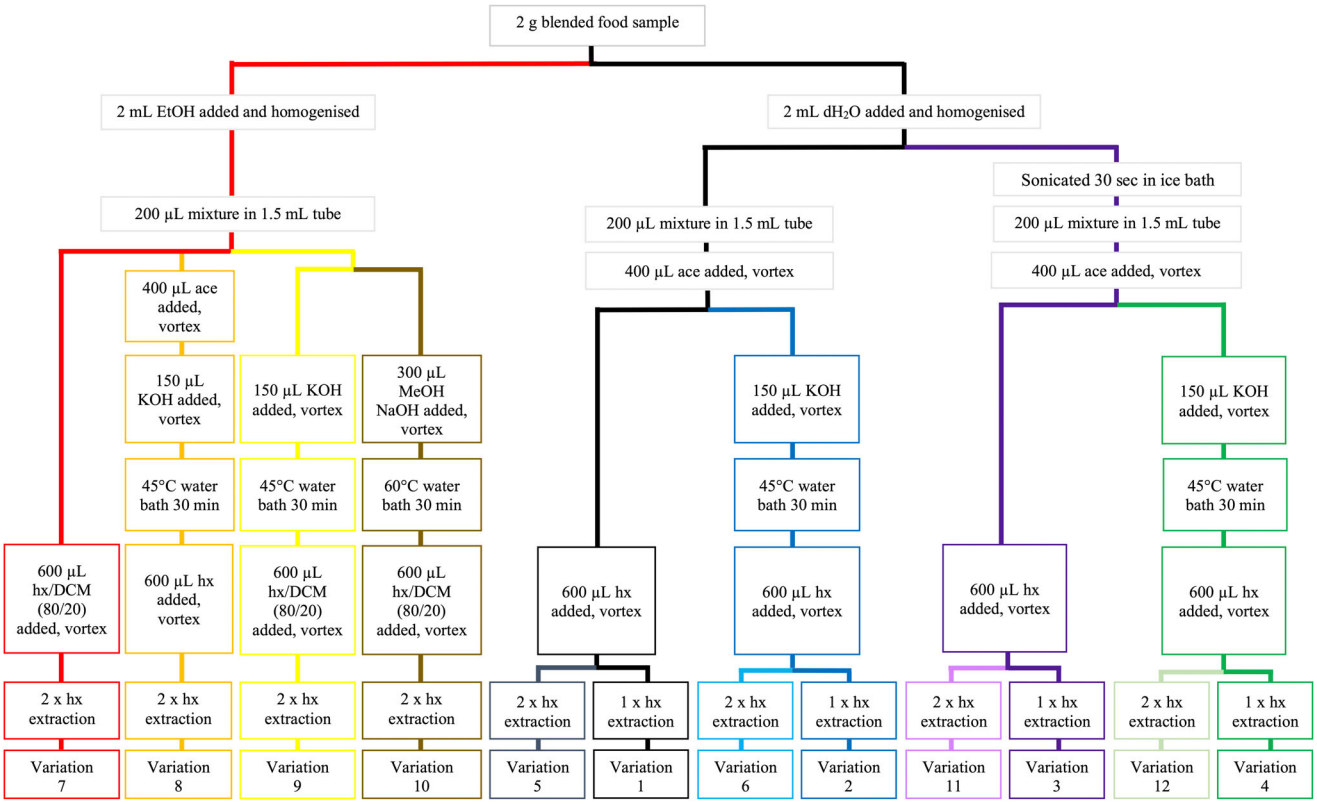

Figure 1 Variations to food preparation and extraction method. ace, acetone; DCM, dichloromethane; dH2O, distilled water; EtOH, ethanol; hx, hexane; KOH, potassium hydroxide; MeOH NaOH, methanol sodium hydroxide.

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

Absorbance was measured at 445 nm for lutein and 450 nm for zeaxanthin. The length of the cuvette was 1 cm. The extinction coefficient (ε) used for lutein was 145 and zeaxanthin 141 (Scott, 2001). The limit of detection at 445 nm for lutein was 0.009 and 0.05 µg/mL for zeaxanthin. Standard curves measured for lutein were linear between the range of 0.009–90 µg/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 200 µL food samples were spiked with 100 µL of 90 µg/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 90 µg/mL lutein standard to obtain a percentage of lutein standard present in the spiked food sample.

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/100 g edible raw food portion for baby orange capsicum, goji berry, and baby spinach, and mean µg/100 g edible cooked food portion for broccoli and broccolini.

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

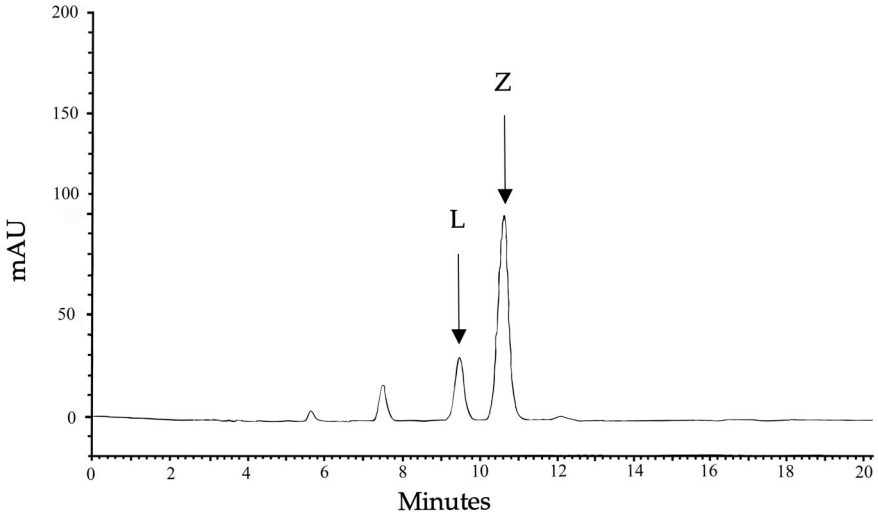

Figure 2 Capsicum, orange, baby chromatogram. L, lutein; mAU, milli absorbance units; Z, zeaxanthin.

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

		Method variation (μg/100 g)			
Sample ID	**Lutein or zeaxanthin**	**1**[a]	**2**	**3**	**4**
1A (*n* 3)*	Lutein	8301 ± 568	6791 ± 254	-	-
	Zeaxanthin	259 ± 29	304 ± 24	-	-
1B (*n* 3)	Lutein	7128 ± 197	6194 ± 228	6947 ± 158	6455 ± 512
	Zeaxanthin	266 ± 5	190 ± 8	262 ± 21	191 ± 16
1C (*n* 3)	Lutein	6842 ± 168	6261 ± 240	6897 ± 132	6025 ± 382
	Zeaxanthin	224 ± 17	166 ± 9	196 ± 8	157 ± 11
1D (*n* 2)	Lutein	8657 ± 2	6914 ± 1576	7231 ± 138	7794 ± 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. *n*, number of replicates analysed per sample.

berries. A chromatogram depicting lutein and zeaxanthin of baby orange capsicum is shown in Fig. 2.

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 2). Refer to Fig. 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 Data S1, broccolini see Data S2, baby orange capsicum see Data S3, and dried goji berry see Data S4.

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 2). 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

Table 3 Baby spinach, Sample 1E, method variation 5, lutein and zeaxanthin obtained per extraction, multiple extractions

	Replicate 1		Replicate 2	
Extraction number	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/100 g)*	12 957	383	13 110	416

*Sum of four extractions.

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. 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.

Testing of multiple hexane extractions

Given the moderate efficiency found from method variations 1 to 4, multiple hexane extractions were tested to improve on the moderate efficiency found from method variations 1–4 (Tables 3 and 4). Method variation 5 was different to variation 1 with two hexane extractions rather than one, and was conducted on Sample E (Table 3). 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.

Analysis of two individually analysed hexane extractions was also conducted for Sample F (Table 4). 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. 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 13 033.5 μg/100 g and the first extraction contributed to 51.6% of this total. In Sample F, the mean total lutein from two extractions was 7992 μg/100 g 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.

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 4).

Table 4 Baby spinach Sample 1F, lutein and zeaxanthin obtained per extraction, multiple method variations

		Replicate 1 (% total)†		Replicate 2 (% total)†		Mean of replicates	
Method variation	Extraction number	Lutein	Zeaxanthin	Lutein	Zeaxanthin	Lutein	Zeaxanthin
5	1	94.9	100	95.9	100	95.4	100
	2	5.1	0	4.1	0	4.6	0
	Combined (μg/100 g)*	8184	228	7800	225	7992	227
6	1	98.1	100	97.9	100	98	100
	2	1.9	0	2.1	0	2	0
	Combined (μg/100 g)*	7427	248	7177	233	7302	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/100 g)*	7536	243	7317	247	7439	245
12	1	95.8	100	95.6	100	95.7	100
	2	4.2	0	4.4	0	4.3	0
	Combined (μg/100 g)*	7236	209	7365	211	7300	210

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

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).

Comparison of method variations 5, 7, 9, and 10

Method variation 5 was compared with variations 7, 9, and 10 using Sample 1G (Table 5). 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. 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 Comparison of method variation 5, 7, 9, and 10 with Sample 1G

Sample ID	Method variation	Mean ± SD lutein (μg/100 g)	Mean ± SD zeaxanthin (μg/100 g)
1G (*n* 7)[†]	5	9270 ± 448	250 ± 24.4[a]
	7	9018 ± 316	261 ± 12.1[a]
	9	10 325 ± 464[b,c]	145 ± 12.1
	10	10 149 ± 441[b,c]	241 ± 13.0[a]

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 zeaxanthin $P < 0.0005$.

[b]Method variation significantly different to variation 5 for lutein $P < 0.005$.

[c]Method variation significantly different to variation 7 for lutein $P < 0.0005$.

Impact of extraction method variations on broccoli, broccolini, baby orange capsicum, and dried goji berry

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 6). Using method variation 9, the mean concentration of lutein in these four foods ranged from 231 μg/100 g to 2386 μg/100 g, and 0 μg/100 g to 2948 μg/100 g of zeaxanthin (Fig. 3). Further detail on lutein and zeaxanthin concentrations measured for the different method variations in these four foods is outlined in Data S1–S4.

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,

Table 6 Optimal variation of extraction method for broccoli, broccolini, baby orange capsicum, and dried goji berry

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%

BDL, below detection limit; *n*, number of replicates analysed per sample.

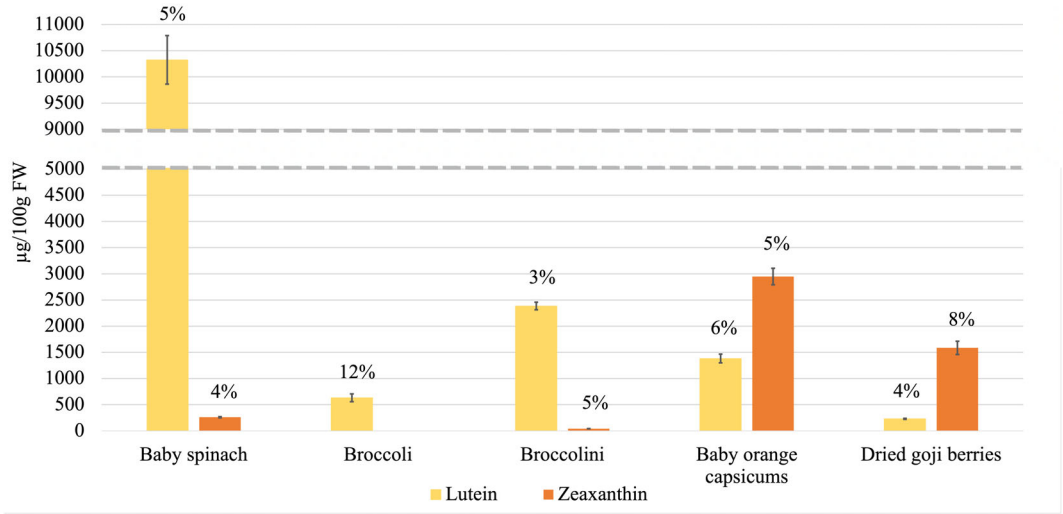

Figure 3 Mean concentration of lutein and zeaxanthin with optimal extraction method variation, variation 9, for broccoli, broccolini, baby orange capsicum, dried goji berry. Error bars indicate standard deviation of the mean. Figure above bar indicates the coefficient of variation as a percentage of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

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.

Influential extraction steps

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 1–4 involved a single hexane extraction. Method variations 5–12 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 6660 µg/100 g in Sample 1E, and 95.4% or 7624 µg/100 g in Sample 1F. The total lutein of all individually analysed extractions combined in Sample 1E was 13 033.5 µg/100 g, 63% more than the total lutein of 7992 µg/100 g 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.

Mixed versus single solution extraction solvents

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 (Agarwal *et al.*, 2020). 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 (Rivera & Canela, 2012).

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 (Agarwal *et al.*, 2020). 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 (Irakli *et al.*, 2011; Watanabe *et al.*, 2011). The addition of a saponification step of 150 μL of 10 molar KOH and incubation in a light protected water bath at 45 °C for 30 min 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.

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 Data S1, Table 2). 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.

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 (USDA, 2018; FSANZ, 2019a). 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 33 μg/100 g and was measured with variation 2. The mean lutein concentration of the nine broccoli samples was 841 μg/100 g (range: 276–1150 μg/100 g), with only one sample reporting a value below the FSANZ reported mean value of 352.5 μg/100 g lutein (range: 0.5–800 μg/100 g) (FSANZ, 2008). The USDA tables report a mean lutein and zeaxanthin value of 1080 μg/100 g (range: 447–1940 μg/100 g) for boiled and drained broccoli (USDA, 2018). 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 100 g 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 2540 μg/100 g (range: 2114–3121 μg/100 g), 79% above the FSANZ reported value for boiled and drained broccolini of 1417 μg/100 g (zeaxanthin not reported) (FSANZ, 2019a). Broccolini is not reported in the USDA tables (USDA, 2018). 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 (Agarwal *et al.*, 2020). In this study, the mean concentration was 523 μg/100 g (range: 170–1384 μg/100 g) for lutein and 697 μg/100 g (range: 167 μg/100 g–2948 μg/100 g) for zeaxanthin. An Australian study of seven orange appearing capsicum varieties measured mean ± SD zeaxanthin concentrations between 1.9 ± 0.1 mg/100 g and 28 ± 8.5 mg/100 g (Agarwal *et al.*, 2020). 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 (Lefsrud *et al.*, 2007; Walsh *et al.*, 2015). The concentrations of zeaxanthin in baby orange capsicums in this study aligns with lower zeaxanthin concentration varieties previously reported for mature orange capsicums (Agarwal *et al.*, 2020). The USDA and FSANZ tables do not report values for orange capsicum or baby orange capsicum (USDA, 2018, FSANZ, 2019a). The USDA tables report a lutein and zeaxanthin value for raw green capsicum of 341 μg/100 g which may underestimate lutein and zeaxanthin intake from baby orange capsicums in Australia by 72%.

The mean baby spinach values were 8905 μg/100 g (range: 6842–13 034 μg/100 g) for lutein and 284 μg/100 g (range: 227–400 μg/100 g) 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 5830 μg/100 g (range: 5320–7110 μg/100 g), and zeaxanthin concentration was 191 μg / 100 g (range: 0–511 μg/100 g) (USDA, 2018). The USDA baby spinach lutein and zeaxanthin values were measured as part of a larger analysis capturing more carotenoids than just lutein and zeaxanthin (Craft, 2001; USDA, 2018). Baby spinach lutein or zeaxanthin is not reported by FSANZ currently (FSANZ, 2019a). 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 (Pennington, 2002; Yates *et al.*, 2021). The observed differences also support the pursuit of a targeted program to develop Australian lutein and zeaxanthin FCTs.

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 (Fitzpatrick *et al.*, 2022). 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.

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Author contributions

Naomi Kathleen Fitzpatrick: Conceptualization; investigation; writing – original draft; methodology; writing – review and editing; visualization; validation; formal analysis; project administration; data curation. **Veronique Chachay:** Conceptualization; methodology; writing – review and editing; supervision; resources; formal analysis; validation. **Angela Shore:** Conceptualization; writing – review and editing; methodology; formal analysis; supervision; validation. **Sarah Jackman:** Conceptualization; writing – review and editing; supervision; validation. **Sandra Capra:** Supervision; formal analysis; visualization; validation; conceptualization; writing – review and editing. **Joanna Bowtell:** Conceptualization; methodology; validation;

supervision; writing – review and editing. **David Briskey:** Writing – review and editing; supervision; methodology; conceptualization; investigation; validation; visualization; formal analysis.

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The authors have no conflicts of interest to declare.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1.
Data S2.
Data S3.
Data S4.
Data S5.