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Abstract

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***Research note*:**

Comparison of solvent regimes for the extraction of photosynthetic pigments from leaves of higher plants

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Abstract. The relative efficiency of methanol- and acetone-based solvents for the extraction of pigments from photosynthetic tissues of plant was compared, together with the advantages of multiple versus single extractions. The two commonly employed triple acetone extractions (100:80:80% and 85:100:100%) performed comparably for most pigments and for all plant species tested. Single extractions with either 96% methanol or 85% acetone failed to extract the more hydrophobic pigments, especially β-carotene. We conclude that multiple extractions that combine pure and aqueous (80–85%) acetone are preferable for extraction of the full range of pigments. These results suggest that previous studies that have utilised aqueous methanol (especially in a single extraction) have probably underestimated the concentration of β-carotene relative to other pigments.

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Keywords: β-carotene, carotenoids, chlorophylls, cycle pigments, HPLC, pigment extraction, xanthophylls.

Abbreviations used: β-car, β-carotene; CAM, Crassulacean acid metabolism; chl, chlorophyll; HPLC, high-performance liquid chromatography; L, lutein; N, neoxanthin.

Introduction

Methodology for extraction of plant photosynthetic pigments lacks consistency despite the growing number of studies in this area. Traditional methods for analysis of photosynthetic pigments employed spectroscopy and extinction coefficients that had been calculated for a range of solvents (Davies 1976; Lichtenthaler 1987; Lichtenthaler and Wellburn 1983; Porra et al. 1989). For whole-leaf extracts these methods allowed for the accurate calculation of chlorophyll (chl) *a* and *b* concentration, but were limited to a pooling of the carotenoid pigments to give total carotenoid content. Although contemporary studies still use these simple, effective and cheaper methods for quantification of chl (Day and Vogelmann 1995; Gehrke 1999; Xiong and Day 2001), high-performance liquid chromatography (HPLC) is now the method of choice when individual carotenoid concentrations are required (Thayer and Björkman 1990; Gilmore and Yamamoto 1991; Wright et al. 1991, 1997; Jeffrey

et al. 1999). For chl extraction from leaves of higher plants several different extractions have been tested and optimal procedures established (Porra et al. 1989). However, this is not the case for extraction of the full range of carotenoid pigments. Therefore, we sought to determine if certain extraction procedures are preferable for efficient extraction of plant photosynthetic pigments. We believe that it is important to elucidate whether or not all commonly employed extraction procedures produce equivalent extraction of the range of pigments and thus allow comparisons between studies undertaken with different extraction procedures. We were concerned that some differences reported in the literature might reflect the extraction procedure rather than an intrinsic difference in the photosynthetic pigments (Robinson et al. 2003).

Since an efficient methodology for HPLC separation of the xanthophyll cycle pigments was developed by Gilmore and Yamamoto (1991), there have been several studies on the levels of photoprotective xanthophyll cycle pigments and the antioxidant carotenoid, β-carotene (β-car; representative list see Table 1). In the majority of these studies pure or aqueous acetone was used as the solvent, often with multiple extractions. Acetone was also traditionally used for extraction of chl for spectrophotometry (Porra et al. 1989). In 1997 Wright and co-workers published a study that investigated the best extraction technique for HPLC of algal pigments (Wright et al. 1997). Sonication with pure methanol was found to be the most effective and safe solvent. Some recent studies have subsequently used an aqueous methanol extraction regime, derived from Wright's methodology, for leaves of higher plants (Table 1).

The methodologies employed can be categorised into two main groups on the basis of the solvent used for extraction, methanol or acetone. The latter group can be divided into two subgroups, those that use pure acetone as the first solvent followed by aqueous acetone (80%; e.g. Lovelock and Robinson 2002), and those that use 85% acetone as the first solvent with subsequent extractions of pure acetone (after Thayer and Björkman 1992). In addition, most of the acetone extractions have been optimised to some extent and two or three extractions are usual. Initially single extractions with 80–85% acetone were used but problems with recovery of β-car led to the adoption of multiple extractions, employing a combination of pure and aqueous (80–85%) acetone (Thayer and Björkman 1992). The main difference between these latter methods concerns the order of aqueous and pure acetone. Some authors use aqueous acetone followed by pure acetone (Adams and Demmig-Adams 1992; Thayer and Björkman 1992) whereas others, including those in our laboratory, have used pure acetone as the first solvent. Previously we have found that an initial extraction with pure acetone was required when working with tissues with a high water content, such as Crassulacean acid metabolism (CAM) succulents (Robinson et al. 1993). With methanol-based solvents, a single extraction is often employed and these methods tend not to follow the comprehensive procedure used by Wright in the original paper (Wright et al. 1997). Although the latter method has been rigorously tested, and methanol confirmed as the solvent of choice for extraction of algal pigments, it has not been tested or optimised for higher plant tissues.

The pigments of concern to Wright and co-workers (Wright et al. 1991, 1997) were those characteristic of algal groups and therefore used in the identification of algal species, such as the fucoxanthins and chl *c*. In studies with higher plant tissues these pigments are absent and other pigments such as the xanthophyll cycle pigments and the carotenes are of most interest to researchers. We were concerned that the carotenes, which are the least polar carotenoids, might be less efficiently extracted by methanol- than acetone-based solvents. We have compared three published methodologies for extraction of photosynthetic pigments utilising methanol (96%) or acetone (85% and 100%) as solvents (Tables 1, 2). Since methods also varied in the number of subsequent extractions that were employed, we compared up to four extractions to determine the most appropriate regime for a range of plant tissues.

Different plant types were tested including examples with relatively simple cellular structure as well as herbaceous, succulent and sclerophyllous leaves. These were an alga (*Ulva* spp.), a moss (*Ceratodon purpureus*), a non-sclerophyllous tree (*Hymenosporum flavum*), a succulent CAM plant (*Cotyledon paniculata*) and a sclerophyllous tree (*Eucalyptus longifolia*). Since initial water content of tissues may be a factor in determining extraction efficiency we compared extraction with the different solvents from a very dry tissue, desiccated moss, and a succulent leaf with high water content.

Materials and methods

Plant material

The thalli or leaves of five different plant types, algae, moss, succulent, non-sclerophyllous and sclerophyllous trees, were collected between 1300 and 1600 h on a sunny day. All plant material was taken from an area on the plant with maximal sun exposure for at least 4 h before sampling, in order to promote conversion of violaxanthin to zeaxanthin. The plant material was immediately placed in liquid nitrogen and stored frozen until extraction.

Two plants of the alga, *Ulva* spp., were collected from a north facing depression at low tide on the rock platform at Wollongong Harbour, NSW, Australia. The uppermost 3 mm of the moss, *Ceratodon purpureus,* was harvested from a turf near the entrance to Wollongong University. Samples of this were also desiccated for 48 h over silica gel. Four leaves were collected from a tree of the non-sclerophyllous Australian native, *Hymenosporum flavum* in Wollongong Botanic Gardens, NSW, Australia. Another four leaves were taken from a sclerophyllous native Australian tree, *Eucalyptus longifolia,* and a succulent CAM plant, *Cotyledon paniculata,* growing in the grounds of Wollongong University.

For the wet moss and algae each sample consisted of 50 mg (± 5 mg) of green, plant material while for *C. paniculata* 300 mg (± 6 mg) of leaf tissue was used. For the dry moss each sample consisted of 5 mg (± 0.1 mg) of plant material. Leaf discs (0.8 cm^2) of *H. flavum* and *E. longifolia* were paired for different extraction regimes, with discs taken from each leaf on opposite sides of the midvein.

Basic extraction methodology and quantification of pigments by HPLC

Plant material (thalli or leaf disks) was weighed, then ground in a mortar and pestle with liquid nitrogen and sand. The first solvent A (1.5 ml; see below and Table 2) was added and the sample ground then transferred to

an eppendorf tube and allowed to stand on ice in the dark for approximately 20 min. After centrifugation (14 000 *g*, 4 min) the supernatant was removed and the pellet was re-extracted with the second solvent B (0.5 ml) using a polypropylene tissue grinder (Crown Scientific, Sydney, Australia). After a further 10 min on ice and centrifugation, this second supernatant was removed and the pellet was re-extracted with solvent B (0.5 ml). Supernatants were combined and samples were made up to an equivalent volume with solvent B. Immediately before HPLC analysis samples were filtered (0.45 µm PTFE syringe filter, Alltech, Sydney, Australia) into amber vials. Samples were kept at –20°C before analysis and were quantified within 24 h of extraction.

Chlorophylls and carotenoids were quantified by HPLC using a method adapted from Gilmore and Yamamoto (1991). Samples extracted with different solvents were alternated to minimise error. Samples (40–100 µL) were injected into the Shimadzu HPLC system [Shimadzu Scientific Instruments (Oceania) Pty Ltd Rydalmere, NSW, Australia] by autosampler (Model SIL-10Ai**,** Shimadzu**)** at a flow rate of 2 ml min-1. Solvent A (acetonitrile : methanol : Tris HCL buffer 0.1 M pH 8.0; 79 : 8 : 3) ran isocratically from 0 to 4 min, followed by a 3-min linear gradient to 100% solvent B (methanol : hexane; 4 : 1) which then ran isocratically from 7 to 14 min. Flow rate was decreased from 2 to 1.5 mL min^{-1} from 7.5 to 12 min and then run at 1.5 mL min^{-1} until 13 min to maintain stable pressure. The column was re-equilibrated with solvent A between samples. Pigments were separated on an Allsphere ODS1 column (Alltech, Sydney, Australia) and quantified by integration of peak areas, detected at 440 nm using a photo diode array detector (Model SPD-M10AVP; Shimadzu) using the Class VP software package (v 5.03, Shimadzu). Concentrations of pigments are expressed as absorbance units on a dry or fresh weight basis.

Comparison of the efficiency of different solvents and numbers of sequential extractions required for extraction of photosynthetic pigments from various plant tissues

Three common extraction regimes were compared, which used either aqueous methanol or acetone / aqueous acetone as solvents. In most cases the methanol extractions are performed as single extractions while the two acetone extraction regimes normally consist of three sequential extractions with various concentrations of pure / aqueous acetone (Table 1). For the purposes of this study, single extractions with 96% methanol, pure acetone and 85% aqueous acetone were compared (Table 2) for the wettest and driest tissues, namely *C. paniculata* leaves and desiccated *C. purpureus*. Triple extractions with either methanol (96% methanol: 4% 0.5 M ammonium acetate pH 7.1) or two acetone / aqueous acetone regimes (100%, 80%, 80% and 85%, 100%, 100%) were also performed for this succulent and the dry moss. The two triple acetone extraction regimes were also compared for the eucalypt, along with a fourth extraction with acetone. Finally the triple methanol and acetone (100%, 80%, 80%) extraction regimes were compared for the alga, wet moss, eucalypt and *H. flavum* leaves.

In order to compare sequential extractions the following protocol was used. After the first extraction with solvent A the supernatant was split equally between three tubes (four tubes, acetone regime, eucalypt only). The second supernatant (solvent B1) was split evenly between tubes 2–3 (and 4, acetone regime eucalypt only). The third supernatant (solvent B2) was added to tube 3 (and 4, as above). A fourth re-extraction with 80% acetone

(B3) was applied to the eucalypt acetone extraction to determine if the three extractions were sufficient, the supernatant from this was added to tube 4. Volumes in all tubes were then made up to 1mL with solvent B.

Statistical analysis

Four replicate samples of each plant tissue were extracted except for the desiccated moss where $n = 3$. Analysis of variance (ANOVA) was used to compare the extraction efficiency of the three solvent regimes and the efficiency of single *v.* multiple extractions for each pigment (violaxanthin, neoxanthin (N), antheraxanthin, chl *b*, lutein (L), zeaxanthin, chl *a*, α-carotene, β-car) for each plant species; [*Ulva* spp., *C. purpureus* (wet and dry), *C. paniculata*, *H. flavum* and *E. longifolia*]. Post hoc Tukey HSD tests were used to determine significantly different pairs. Statistical tests were performed using the JMP statistical package (v4.0 SAS Institute Inc. Cary, NC).

Results

Comparison of the number of extraction steps

Single extractions were less efficient than triple extractions for several pigments in both the succulent leaves (Fig. 1) and desiccated moss tissue (Fig. 2). For the succulent plant the chl (*a* and *b*) and the less polar carotenoids (L and β-car) had significantly higher concentrations in the third extraction than the first extraction (Table 3) for most solvents. The only exception to this was that a single extraction with pure acetone extracted β-car as well as the triple extractions with acetone (see interaction term, Table 3). For succulent tissues the triple methanol extraction improved the extraction of all pigments compared with the single extraction. For the acetone regimes there was little improvement between the first and third extractions when pure acetone was the first solvent, however if 85% acetone was applied first, then the subsequent extractions with 100% were required to remove the less polar pigments such as chl (*a* and *b*) and β-car. For the desiccated moss chl *a* and β-car were more efficiently extracted by the triple extraction regimes (Table 3). The efficiency of extraction from the eucalypt leaves was also tested using the acetone (100%, 80%, 80%, 80%) regime, in this case the main improvement was seen between the first and third extractions (Table 4) and the fourth extraction produced little improvement (data not shown) although the increased concentration of pigments in subsequent extractions (1–4) was not significant.

Comparison of methanol- and acetone-based solvents

Acetone is a better solvent for the least polar carotenoids and the chl (*a* and *b*; Figs 1, 2, Table 3). For the succulent leaves, aqueous methanol extracted less of both chl (*a* and *b*) and β-car (Fig. 1). For the desiccated moss, aqueous methanol extracted less chl *a*, L and β-car (Fig. 2). In most cases the two acetone extraction regimes worked with similar efficiency. The exception to this was with β-car, which was extracted better when 100% acetone was the first solvent (significantly for the first extraction in the succulent leaves, see above, and overall for the desiccated moss).

Triple extractions with acetone (100%, 80%, 80%) and methanol (96%) were also compared for four other plant tissues *Ulva* spp., *C. purpureus* (wet moss), *H. flavum* and *E. longifolia* The only pigment that was significantly affected by extraction regime in this case was β-car which was lower with the triple methanol extraction for all the species tested (*Ulva* spp., $F_{1,6}$=10.2135, *P*=0.0187; *C. purpureus* $F_{1,6}$=11.0062, *P*=0.0161; *H. flavum* $F_{1,6}$=4.6116, *P*=0.0754; *E. longifolia* $F_{1,6}$=12.4064, *P*=0.0125; Fig. 3).

Discussion

The pigments that are most affected by solvent regime are the two chl and the less polar carotenoids, particularly β-car. The aqueous methanol and acetone do not appear to extract these hydrophobic pigments as efficiently as acetone. The polar carotenoids such as N showed very little response to either solvent regime or number of extractions.

Overall these results show that the least polar pigments, particularly β-car, are inefficiently extracted by aqueous methanol or acetone in a single extraction. Single extraction regimes for acetone are not common in the literature for higher plants but single methanol extractions are. Triple extractions involving 100% acetone were initially developed by Thayer and Björkman (1992) because extraction of β-car with aqueous acetone was inadequate. Our results show that methanol is an even poorer solvent for the hydrophobic pigments. If only single extractions are employed pure acetone is probably preferable to the other alternatives, especially for tissues with high water content.

These results show that triple extractions with aqueous methanol are effective for most pigments with the important exception of β-car. However, if we compare the commonly published methods (single aqueous methanol *v.* either of the triple acetone regimes) the results for the aqueous methanol extraction are very unsatisfactory and would underestimate the concentrations of several pigments especially the less polar chl and carotenoids. Regardless of solvent used, multiple extractions are preferable to achieve a representative extraction of the various pigments. For many tissues, two extractions will be sufficient (incorporating pure acetone and an aqueous acetone), but this should be tested when establishing a method for a new species. This study confirms that results obtained for single aqueous methanol extractions of higher plant tissues should be interpreted with caution especially in regard to the chl and less polar carotenoids.

Although the aqueous methanol extraction regime is loosely based on that devised by Wright et al. (1997), it differs in the way that it has been applied to higher plants. Wright and co-workers employed sonication in pure methanol to extract pigments from alga and this method was mainly used to separate the range of pigments for identification of different algal classes, or groups of species, occurring in field samples of phytoplankton populations. It should be noted that the pure methanol used in these algal studies would be expected to be more efficient than the 96–98% methanol used in the higher plant studies. In addition, sonication is not usually employed in the latter studies, a factor that could further reduce the efficiency of extraction. It is also likely that β-car concentration was less

important in these phytoplankton studies than it is with many higher plant studies. Finally, photosynthetic pigments of higher plants are generally found in complex multicellular, eukaryotic tissues that may require more stringent extraction techniques than single cells. Methanol was also preferred in the phytoplankton studies because it is less flammable than acetone and was therefore a safer solvent to use on board ship. This safety concern therefore needs to be weighed against extraction efficiency.

Although this study was concerned with the initial extraction of pigments from plant tissues, the subsequent storage and analysis of such solutions can also present problems. If samples are stored in vials at low temperature after filtration there is a danger of the pigments precipitating. This problem is easily identified since such aggregates and pigment particles do not bind to the column and will elute before N and violaxanthin. Also for accurate quantification it is obviously important not to overload the HPLC column. Chlorophyll concentrations of 20–40 μmol L^{-1} and 5–50 μL injection for a standard 25 cm ODS-1 column are advised. If in doubt serial dilutions can be performed to ensure that the carrying capacity of the column is not exceeded.

In conclusion, this study has shown that single and even triple aqueous methanol extractions fail to recover all β-car pigments. β-carotene is an important photosynthetic pigment, which often accumulates in leaves under excess light stress. For studies in which these hydrophobic carotenes are of interest, optimum extraction requires pure acetone, as shown by Thayer and Björkman (1992). Our results confirm that optimum extraction of all pigments requires a sequential extraction regime that includes both pure acetone and aqueous (80–85%) acetone.

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Fig. 1. Comparison of pigment concentrations in *C. paniculata* extracted using methanol (96%) and acetone (85% and 100%)in single (open bars) and triple (hatched bars) extractions, as in Table 2. A representative selection of pigments, covering a range of polarities, is shown. Abbreviations, Neoxanthin (N), chlorophylls *a* (chl *a*) and *b* (chl *b*), lutein (L) and β-carotene (β-car). Data represent mean (± s.e.m., $n = 4$).

Fig. 2. Comparison of pigment concentrations in desiccated *C. purpureus* extracted with methanol (96%) and acetone (85% and 100%) in single (open bars) and triple (hatched bars) extractions, as in Table 2. A representative selection of pigments, covering a range of polarities are shown, N is missing because low quantities were recovered from all samples. Abbreviations as in Fig. 1. Data represent mean (± s.e.m., $n = 3$).

Fig. 3. Comparison of pigment concentrations in *Ulva* spp., *C. purpureus, H. flavum* and *E. grandiflora* extracted with triple extractions of 96% methanol (open bars) and acetone (100 : 80 : 80%; hatched bars) as in Table 2. A representative selection of pigments, covering a range of polarities are shown. Abbreviations as in Fig. 1. Data represent mean (± s.e.m., $n = 4$), note different *Y*-axes.

Table 1. Solvents used and number of extractions employed to extract photosynthetic pigments for HPLC analysis from a range of plant tissues

Solvent	Number of extractions	Plant species studied	References
Methanol-based			
MeOH with 2–4% ammonium acetate buffer (0.5M, pH 7.1)	1	*Turgidosculum complicatulum* (lichen), *Prasiola crispa* (algae), *Deschampia antarctica*	(Lud et al. 2001; Lud et al. 2001)]
100% MeOH	4	*Lycopersicon esculentum*	(Ayari et al. 2000)
		Andreaea reularis (moss)	(Newsham 2003)
MeOH:acetone:H_2O (80 : 15 : 5)	1	*Cephaloziella varians* (liverwort), *Sanionia uncinata* (moss)	(Newsham et al. 2002)
Acetone -based			
Acetone	1	*Pisum sativum*	(Jahns and Miehe 1996)
		Quercus ilex	(Llorens et al. 2002)
Acetone 100%	1	Range of semi-deciduous and sclerophyll trees	(Kyparissis et al. 2000; Manetas et al. 2003)
		Arabidopsis	(Russell et al. 1995)
		4 native Australian rainforest trees	(Watling et al. 1997)
Acetone 100%	2	*Anthurium andraenum, Lactuca sativa*	(Gilmore and Yamamoto 1991)
		Arabidopsis	(Müller-Moulé et al. 2002)
		Eucalyptus nitens	(Close et al. 2001)
Acetone 100%	3	*Cotyledon orbiculate*	(Robinson et al. 1993)
		Range of sun and shade leaves	(Krause et al. 2003)
Acetone 100%	multiple	*Cecropia obtusifolia*	(Searles et al. 1995)
Acetone 100% then 80%	2	*Bryum pseudotriquetrum, Ceratodon purpureus, Grimmia antarctici* (mosses)	(Lovelock and Robinson 2002)
Acetone (90%) then 100% twice	3	*Ligustrum ovalifolium*	(Brugnoli et al. 1994)
Acetone 85% twice	2	Range of sun and shade leaves	(Thayer and Björkman 1990)
Acetone 85% twice then 100%	3	*Gossypium hirsutum. Zea mays*	(Thayer and Björkman 1992)
Acetone 85% twice then 100% twice	4	*Helianthus annuus*, *Cucurbita pepo*, *Cucumis sativus, Euonymus kiautschovicus, Malva neglecta*	(Adams and Demmig-Adams 1992)
Acetone 80% with 100% added	1	*Amyema miquelii*	(Matsubara et al. 2001)
N′N′-dimethylformamide	1	*Arabidopsis*	(Pogson et al. 1998)

Table 2. Composition of solvents used to extract photosynthetic pigments from a variety of plant tissues

*solvents B1 and B2 are identical, number refers to sequential extractions

Method	Solvent A	Solvent B1	Solvent B2*	Reference
96% methanol	96% methanol with 4% ammonium acetate buffer (0.5 M, pH 7.1), thrice			(Lud et al. 2001)
85% acetone	85% acetone (1 : 1500 w / v $NaHCO_3$)	100% acetone	100% acetone	(Adams and Demmig-Adams 1992; Thayer and Björkman 1992)
100% acetone	100% acetone (1 : 1500 w / v $NaHCO_3$)	80% acetone	80% acetone	Lovelock and Robinson 2002

Table 3. Summary table for the two-way ANOVA comparing the pigment extraction efficiency of the aqueous methanol and two acetone extraction regimes (solvent regime) and the number of consecutive extractions (extraction number) for *C. paniculata* (Fig. 1) and desiccated *C. purpureus* moss (Fig. 2)

Pigments are shown only where significant effects were found (ns = not significant)

	Extraction number	Solvent regime	Extraction*solvent
C. paniculata			
Chlorophyll *b*	$F_{1,18}$=9.19, *P*<0.0072	$F_{2,18}$=6.10, *P*<0.0095	ns
Lutein	$F_{1,18}$=5.78, *P*<0.0272	ns	ns
Chlorophyll *a*	$F_{1,18}$=8.61, *P*<0.0089	$F_{2,18}$=8.97, *P*<0.002	ns
β-carotene	$F_{1,18}$=27.90, *P*<0.0001	$F_{2,18}$=20.11, *P*<0.0001	$F_{2,18}$=8.13, *P*<0.003
C. purpureus			
Lutein	ns	$F_{2,12}$=7.73, *P*<0.0068	ns
Chlorophyll *a*	$F_{1,12}$=4.26, *P*<0.061	$F_{2,12}$=3.0173, *P*<0.0868	ns
β-carotene	$F_{1,12}$=10.39, *P*<0.0073	$F_{2,12}$=9.35, *P*<0.0036	ns

Table 4. Concentration of chlorophylls and carotenoids extracted from *E. longifolia* leaves using the two acetone extraction regimes (see Table 2). Data represent mean ± s.e.m (*n* = 4)

Solvent regime (acetone concentration)	Single extraction (100% acetone)	Triple extraction (100 : 80 : 80% acetone)	Triple extraction (85 : 100 : 100% acetone)
		Concentration of pigment (Au. g^{-1} fw)	
Neoxanthin	2.117 ± 0.165	2.281 ± 0.159	2.367 ± 0.120
Chlorophyll *b*	5.212 ± 0.455	5.331 ± 0.463	5.574 ± 0.385
Lutein	6.736 ± 0.444	7.344 ± 0.454	7.447 ± 0.393
Chlorophyll *a*	17.724 ± 1.158	18.608 ± 1.140	19.353 ± 0.948
β-carotene	3.599 ± 0.203	3.518 ± 0.149	4.267 ± 0.194