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REVIEW PAPER
A review of carotenoid analysis techniques in the Sri Lankan context and solution to some problems therein

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Abstract

Carotenoid analyses results are subject to much variation. In this study, problems of sampling, extraction, chromatography, spectral data, and chemical tests are discussed together with *in-vitro* bioaccessibility. In addition, problems in the *in-vitro* trolox equivalent antioxidant capacity assay will be discussed. Identifying carotenoids requires *uv/vis* spectra, spectral databases, chromatographic behavior on thin layer chromatography (Tlc), high performance liquid chromatography (HPLC), open column chromatography (OCC) and chemical tests all of which must be used in conjunction. Quantification can be done by OCC and HPLC but correction factors are needed in the latter.

Introduction

Vitamin A deficiency is one of the major deficiency disorders in Sri Lanka (Medical Research Institute, 1998). The populations more subject to the deficiency are low-income groups, which cannot afford to purchase animal derived foods. Therefore plant derived foods, which are rich in pro-vitamin A carotenoids are recommended. For this purpose a reliable database is necessary for the use of nutritionists in recommending appropriate diets. Unfortunately there are many disagreements not only in Sri Lanka but also Internationally (Rodriguez-Amaya, 1999) on collection of data and their interpretation. It appears that in the first instance reliable techniques must be used in both sampling and analyses. The objective of this review is to focus on the problem areas of data generation and interpretation. This includes raw material collection, its appropriate definition of applicable location of collection. Thereafter methods of extraction, identification and quantification need to be carefully detailed. Further, calculation of potential bioavailability (bioaccessibility) and calculation of retinol equivalent (RE) and retinol activity equivalent (RAE) need discussion.

Variation in Raw Material

Raw material must reflect the status of the carotenoid bearing plant in a district, province, area based on rainfall, dominant area of production or prevalence of vitamin A deficiency and this must be explicitly stated in the context of location of raw material collection. Table 1 shows some results on Sri Lankan fruits showing a vast variation that could reflect sampling, genetic, climatic and edaphic factors. Maturity can play a major role as in *Lasia spinosa* stem (*kohila ala*) (Priyadarshani and Jansz, 2006).

In analysing samples for carotenoids two extremes have been found. First in crops such as *Lasia spinosa* stem, papaw and jakfruit there are no agricultural selections in Sri Lanka. Therefore results of random sampling are affected not only by environmental factors but also by genetics and maturity. For example; range of β-carotene for *Lasia spinosa* stem was found to be from 0.9 to 7.2 $\mu g.g^{-1}$ fresh weight (FW) (8 fold variation) (Priyadarshani and Jansz, 2006), while for yellow-fleshed papaw from 1.4 to 10.3 $\mu g.g^{-1}$ FW (~ 7 fold variation) (Priyadarshani *et al.*, 2006) making it virtually impossible for a nutritionist to predict retinol equivalent (RE) per meal/portion. On the other hand, when specially bred agricultural cultivars (where there is no genetic effect) are analysed the diversity of carotenoid content was seen to be much less and standard deviation (SD) could be calculated in species with specific agricultural varieties (cultivars); eg. β-carotene content of carrot (variety: "New kuroda"), pumpkin (variety: "Arjuna") and sweet potato (variety: "CARI-426") were 43.8±5.6, 50.9±5.7 and 42.8±4.3 $\mu g.g^{-1}$, respectively on the basis of fresh weight (Priyadarshani and Chandrika, 2007) but variations were observed within the varieties of the same species eg. β-carotene contents of varieties "Arjuna", "Meemini" and "Ruhunu" of pumpkin were 332.7±37.3, 53.4±18.1 and 113±15.6 $\mu g.g^{-1}$ on dry weight basis (Priyadarshani *et al.*, 2005). Since the consumer rarely purchases agricultural varieties and due to variations in carotenoid content, prediction of percent contribution of a portion to recommended daily allowance (RDA) is very difficult especially in the case of non-agricultural varieties. Further in rare plants species, identification is important. For example lavalu analysed by Chandrika (2004) and Abeyesekara (1993) state that the species is *Chrysophyllum roxburghii* but this species is a green fruit with a white pulp (Dassanayake, 1995) and de Lanerolle (2006) had shown the common lavalu plant to be *Pouteria campechiana* by comparing National Herbarium specimens and authentic identification by an authority.

It is easy to see why it is an International book states that carotenoid analyses in different laboratories are different (Rodriguez-Amaya, 1999).

Table 1: Retinol equivalent (RE) dependent on area

Fruit	Reference	Area	RE ($100g^{-1}$ dry weight)
Papaw (Yellow-fleshed)	Chandrika *et al.* (2003)	Not given	151.6±34.2
Papaw (Yellow-fleshed)	Priyadarshani *et al.* (2006)	Kurunegala District	344.8 to 2410
Lavalu	Abeysekara, (1993)	Colombo District	1867
Lavalu	Chandrika *et al.* (2005a)	Kadawata, Piliyandala Districts	68*
Lavalu	Chandrika (2004)	Kadawata, Piliyandala Districts	23*
Lavalu	de Lanerolle (2006)	Kandy, Kurunegala, Gampaha, Colombo Districts	13 to 3900
Jakfruit	Chandrika *et al.* (2005b)	Colombo suburbs	141.6
Jakfruit	Priyadarshani *et al.* (2007a)	Kurunegala, Matale Districts	Trace amounts to 10
Palmyrah fruit	Pathberiya and Jansz (2005)	Mannar District	0 to 44.5
Palmyrah fruit	Wijemanne *et al.* (2006)	Kalpitiya	75 to 969
Palmyrah fruit	Chandrika (2004)	Hambantota District	159.1

* Same sample

- Note that recent studies with extensive sampling give large variations in range, which is not seen in the work of Chandrika (2004).

Extraction Techniques

The normal procedure for extraction is to cut up the sub-samples, homogenize and extract with acetone until the extract is colourless and filtration with suction. A study has shown that this does not work for lavalu as the multi-seeded type, in particular, has a high gum content that precipitates with acetone and this makes extraction difficult and filtration of carotenoids impossible. This step is made easier after removal of gum by water (de Lanerolle, 2006). The next step is the partition of carotenoids into petroleum ether. Studies have shown some yellow remaining in the water layer, which can be misinterpreted as water-soluble non-carotenoids but this is often due to polar carotenoids (Priyadarshani *et al.*, 2007a). However, re-extraction into petroleum ether to which 10% diethylether is added, removes all colour to the organic layer. The

concentration of diethylether is dependent on the extent of polar carotenoids (Priyadarshani *et al.*, 2007a; de Lanerolle, 2006). Saponification is needed when fat content and hydroxy compounds are high. In such cases carboxylic acid carotenoids (eg. crocetin) will appear in the alkaline water extract (Priyadarshani *et al.*, 2007a) and not in the petroleum ether layer as stated by Chandrika (2004). Saponification should not be done unless there is evidence of fatty acid derivatives of carotenoids (Rodriguez-Amaya, 1999). Fatty acid derivatives can be tested by open column chromatography (OCC) and high performance liquid chromatodraphy (HPLC) (Priyadarshani *et al.*, 2007a) A suitable internal standard must be used in HPLC.

Column Chromatography

The celite:MgO (1:1) method (Rodriguez-Amaya, 1999) is best when used for OCC. This is one matrix as used by Chandrika (2004). It causes problems with medium pressure liquid chromatography (MPLC) (Pathberiya, 2005 unpublished results). Silica gel MPLC as used by Chandrika *et al.* (2003) should not be used as carotenoids are known to decompose on SiO_2 gel (Rodriguez-Amaya, 1999). In OCC, a high suction needs to be applied at the lower end of the column. As solvents are very volatile the collection flask needs to be cooled in ice to improve the rate of elution and to collect sufficient fractions for spectral studies when the temperature is high. Petroleum ether B.P.40-60 °C is especially prone to the problem but using petroleum ether B.P.60-80 °C though lessening volatilation, is more difficult to flush out with nitrogen at a later stage (Priyadarshani *et al.*, 2008).

Spectral Studies

The spectral fine structure is obtained by using a double beam spectrophotometer and a tentative identification can be achieved by comparing λ_{max} at three wavelengths and spectral fine structure using given solvent in the database (Rodriguez-Amaya, 1999). Some Sri Lankan vegetable carotenoids not being listed in databases eg. *Lasia spinosa* stem (Priyadarshani and Jansz, 2006), palmyrah (Pathberiya and Jansz, 2005 ; Wijemanne *et al.*, 2006), jakfruit (Priyadarshani *et al.*, 2007a) poses problems as there are no spectral information for identification and $A^{1\%}_{1cm}$ values are not available for calculation using OCC. In this case it may be convenient, if not strictly accurate, to take the $A^{1\%}_{1cm}$ value of a known compound that elutes close to the unknown carotenoid as it generally often has some common structural features (Pathberiya, 2005).

No problem has been found in detecting epoxides by the epoxy-furanoid rearrangement test by spectral studies; for mono-epoxy, a hypsochromic shift by about 20 nm and for di-epoxy, a hypsochromic shift of about 40 nm from

the major peaks (Rodriguez-Amaya, 1999). However *cis-trans* photo-isomerisation using iodine (I_2) has not been described completely (Rodriguez-Amaya, 1999; Chandrika, 2004). The base-line correction of the spectrophotometer should be done with the addition of exactly the same quantity of iodine into the blank after the isomerisation (Priyadarshani and Jansz, 2006). This is needed as the spectrum of iodine has some overlap with carotenoid spectra. Omission of this step always gives a bathochromic shift by 3-5 nm indicating a *cis* carotenoid (Wijemanne, 2006). In addition the test does not work when the carotenoid concentration is too high when I_2 is not a catalyst but become a reactant and a green colour appears (de Lanerolle, 2006). The reaction has not been explained so far.

The authors are not familiar with problems of allylic methylation and acetylation. However methylation of the dicarboxylic acid crocetin does not work as H_2SO_4 adds onto the double bonds conjugated to the COOH carbonyl and renders the carotenoid colourless (Priyadarshani *et al.*, 2007a).

Thin layer chromatography (Tlc) data

The conventional method of Tlc is 5% methanol in toluene using silica gel plates. Errors arise due to (i) not running the plate to the full extent, (ii) not mentioning activation or non-activation and (iii) not stating the type of SiO_2 gel used; all as seen in the case of Chandrika (2004). To obtain best results pre-prepared, activated plates must be used defining the thickness of SiO_2 gel and its G number. R_f values of some of the carotenoids are given in Table 2.

Table 2: RF values of some carotenoids in 5% methanol in toluene

Carotenoid	R_f values			
	Priyadarshani *et al.* (2008)[a]		Rodriguez-Amaya (1999) Azevedo and Rodriguez-Amaya (2005)[b]	Chandrika(2004) Chandrika *et al* (2005b)[c]
	Non-activated plate	Activated plate		
β-Carotene - HC	0.98	0.98	0.99	1/0.98
α-Carotene - HC	0.97	0.97	Runs with the solvent front	1/0.98
Lycopene - HC	0.97	0.98	Runs with the solvent front	0.98
ζ-Carotene - HC	097	0.96	Runs with the solvent front	0.90
β-Cryptoxanthin-1OH	0.26	0.53	0.44	0.48
Lutein – 2OH	On the origin	0.17	0.21	0.29
Violaxanthin – 2OH	On the origin	0.09	0.12	0.38
Neoxanthin – 3OH	On the origin	0.07	0.07	On the origin
Crocetin – $2COO^-$	ND	0.09	-	0/0.33

[a] Using SiO_2 gel G_{60} plates with thickness of 100 μm – Merck (Priyadarshani *et al*, 2008)

[b] Whether plates are activated or not, type of silica gel are not mentioned (Rodriguez-Amaya, 1999; Azevedo and Rodriguez-Amaya, 2005)
[c] Whether plates are activated or not, type of silica gel are not mentioned. R_f values were calculated from Tlc diagrams (Chandrika *et al.*, 2005b; Chandrika, 2004)
ND – Not determined, HC – Hydrocarbon carotenoid, OH – Hydroxy carotenoid,
COO^- - Carboxylic acid carotenoid

The difference in R_f on activated and non-activated plates is due to moisture absorption on the SiO_2 gel in non-activated plates. This holds back compounds with polar groups and separation is not good for those compounds. However, non-polar compounds separate better. Activated plates give good separation of polar compounds. R_f was shown to be inversely proportional to number of hydroxy groups (Priyadrshani *et al.*, 2008). For aiding identification, authentic carotenoids should be spotted on the same plate with the unidentified carotenoids. On the other hand as different carotenoids give the same R_f value (Priyadrshani *et al.*, 2007a), Tlc alone cannot be used for the identification of carotenoids. This should be done in conjunction with spectral data, chemical tests, HPLC peak enrichment, etc.
Tlc plates can also be used to differentiate non-epoxy, mono-epoxy and di-epoxy groups by exposing carotenoids into fuming HCl (Rodriguez-Amaya, 1999). This works well giving green for mono-epoxy and blue for di-epoxy carotenoids.

High performance liquid chromatography (HPLC) separation
Some studies do not use an internal standard (Chandrika, 2004; Chandrika *et al.*, 2005a). This exposes results to questioning, as use of internal standard is not only as correct for recovery but also for verification of authenticity of results even if there is auto-injection. A pre-requisite for HPLC separation is to identify the peaks. Relative retention time is not enough as carotenoids can co-chromatograph eg. an unidentified carotenoid in sweet potato had the same retention time with that of lutein (Priyadarshani *et al.*, 2007b) and β-cryptoxanthin and violaxanthin in lavalu co-chromatographed (de Lanerolle, 2006) in the traditional mobile phase solvent system (acetonitrile:methanol:tetrahydrofuran, 58:35:7) and in the column of spherisorb ODS_2 5 μm monomeric C_{18} with 250 mm in length. In this case the ratio of the compounds that co-chromatograph in HPLC can be calculated by OCC (de Lanerolle, 2006). This solvent system of HPLC can be changed but it leads to other complications. In any case peak enrichment by standards or identified OCC isolates is important.

Quantification
There are two methods of quantification by OCC and by HPLC (with internal standard). Calculation by OCC is straightforward, but it is more time consuming. Here calculation is as follows (Rodriguez-Amaya, 1999).

$$x\,(\mu g) = \frac{A \cdot y\,(mL) \cdot 10^6}{A^{1\%}_{1cm} \cdot 100}$$

Where,

x = Weight or the concentration of the carotenoid
y = Total volume of the carotenoid solution
A = Absorbance at a specified wavelength
$A^{1\%}_{1cm}$ = Absorption coefficient of the carotenoid used

The problem arises with unidentified carotenoids where $A^{1\%}_{1cm}$ is not known. Here chemical and Tlc tests usually give a good clue to the type of carotenoid. In these cases an approximate calculation given earlier can be made (Pathberiya and Jansz, 2005; Priyadarshani and Jansz, 2006).

Photodiode array (PDA) detectors can be used to check for cross contamination in HPLC and also give the absorption for the λ_{max}. However in Sri Lanka most HPLCs do not have this facility. The PDA is not the panacea for all ills such as cross contamination as the PDA gives spectra of compounds in the mobile phase of HPLC which is not the same as for solvents specified in databases. Thus the researcher has two options available. Firstly in the case of simple profile (2-4 compounds) the λ_{max} of each can be scanned. Usually this is not the case as the number of carotenoids can be very high eg. *Lasia spinosa* (8 for type A plant), palmyrah (8) and jackfruit (8) scanning at each λ_{max} is not convenient. In this case another option is to obtain the ratio of absorbance at 450 nm (normal scanning λ) and the λ_{max} and multiplying by a conversion factor. Table 3 gives some of these conversion factors.

i.e. $\frac{A \text{ at } \lambda_{max}}{A \text{ at } 450 \text{ nm}}$

Where,
A = Absorbance of the carotenoid, λ_{max} = Maximum absorption

It is recommended that each researcher should work out the correction factor from spectra of their pure isolates.

Table 3: Conversion factors for λ_{max} difference in HPLC

Compound	Conversion factors[a] (Multiply by)
Phytofluene	9.0
ζ-Carotene	7.6
Violaxanthin	1.2
Neoxanthin	1.5
Lycopene	1.6
Lutein	1.1
α-Carotene	1.1
β-Carotene	1.0

[a]Priyadarshani *et al.* (2008) ; de Lanerolle (2006)

This too is not enough as molar absorption coefficient at λ_{max} is also different from that of 2 used. An other correction factor to be used will be:

$$\frac{A^{1\%}_{1cm} \text{ at } ^{\lambda_{max}} \text{ of compound}}{A^{1\%}_{1cm} \text{ for } \beta\text{-carotene}}$$

$A^{1\%}_{1cm}$ = Absorption coefficient of the carotenoid, λ_{max} = Maximum absorption

This needs to be done even if a PDA is available. Fortunately, except in a few cases the values do not change much (Rodriguez-Amaya, 1999). In all cases there needs to be an internal standard which is usually β-apo-8' carotenal (*trans*) (Priyadarshani and Jansz, 2006). In some cases an internal standard has not been used (Chandrika, 2004).

Standard curves can also be used for each compound (Chandrika, 2004) and here conversion factors are not needed. However, standard curves will not give an idea of recovery and is better used when there is auto-injection though it is still prone to error (Rodriguez-Amaya, 1999). Plotting a standard curve on each day of analyses can minimize the error.

The *in vitro* bio-accessibility assay (Hedren *et al.*, 2002)

In this assay the shortcoming is for plants with a tough matrix eg. lavalu (de Lanerolle, 2006) and jakfruit where cutting alone will not mimic masticating, in which there are varying degrees of 'pounding' action (Priyadarshani *et al.*, 2007a). For jakfruit the strict *in-vitro* assay gave 8% bioaccessibility but on introducing an *iv-vivo* mastication step by individuals gave values of 12, 14 and 18% (Priyadarshani *et al.*, 2007a). This shows the effects of the pounding action of mastication on the results of *in-vitro* bioaccessibility technique. Studies reveal that in the *in-vitro* procedure reagents are not limiting (Priyadarshani *et al.*, 2006). This is probably due to the fact that this procedure was worked out for carrot (Hedren *et al.*, 2002), which has a very high carotenoid content.

Retinol Equivalent (RE) and Retinol Activity Equivalent (RAE)

One retinol equivalent (RE) is defined as being equivalent to 6 μg of β-carotene and 12 μg of other carotenoids (FAO, 1999). "Other carotenoids" need to be re-defined as 12 μg of pro-vitamin A carotenoids depending on structure, as carotenoids without a β -ionone ring will not produce vitamin A.

There is also in vogue, a term called "retinol activity equivalent" (RAE) (Hedren, 2004) which should have the same qualification (i.e. pro-vitamin A carotenoid) in the definition as being equal to 12 μg of β-carotene and 24 μg for other pro-vitamin A carotenoids.

RAE is expected to take into account the matrix effect. The two calculations were used for papaw and jakfruit and the following emerged.

Papaw; (Priyadarshani *et al.*, 2006)
RE = 3.13 g^{-1} FW
RAE = 1.57 g^{-1} FW
Percentage *in-vitro* bio-accessible (% IVB) β-carotene = 51
Here RAE = RE × % IVB /100
Where, FW = Fresh weight

Jakfruit; (Priyadarshani *et al.*, 2007a)
RE = 10 per100g DW
RAE = 5 per100g DW
Percentage *in-vitro* bio-accessible (% IVB) β-carotene = 8
Here RAE ≠ RE × % IVB /100
Where, DW = Dry weight

This is clearly due to the difference in the matrix of these two fruits, jakfruit having a much larger matrix effect which is shown by RAE not being equal to RE × % IVB /100 as in the formula.

In-vitro carotenoid antioxidant problems

Determination of *in-vitro* antioxidant capacity using $ABTS^{+\cdot}$ (2, 2'- azino-bis (3-ethylbenzothiazoline 6-sulphonic acid) cation free radical diammonium salt) in aqueous medium (Awika *et al.*, 2003) is possible only in very dilute carotenoid solutions. Pathberiya and Jansz (2005) showed that the trolox (vitamin E analogue) equivalent antioxidant activity (TEAC) of palmyrah carotenoids is proportional to the extent of conjugated double bonds but the absolute value may be subject to some errors due to the extent of dilution. The $ABTS^{+\cdot}$ method in ethanolic medium (Huang *et al.*, 2005) allows higher concentrations of carotenoids to be used but the effect of carotenoid concentration is not proportional to the *in-vitro* antioxidant activity at high carotenoid concentrations (Priyadarshani *et al.*, 2008). This may be due to the special mechanism of action of carotenoids as antioxidants *viz.*, activation of carotenoid free radical to high energy state. This at a high concentration of carotenoid may allow transfer of the free radical of one carotenoid molecule to another carotenoid molecule. However the trolox antioxidant potential of isolated carotenoids showed lycopene>β-carotene$\geqslant\alpha$-carotene>β-cryptoxanthine $\geqslant$ lutein with p value <0.05 with respect to lycopene and β-carotene (Priyadarshani *et al.*, 2008). Thus, giving a good agreement with the structure of carotenoids and *in-vitro* antioxidant potential. It is concluded that the *in-vitro* method of assessing individual carotenoid antioxidant potential is not ideal. Therefore researchers need to resort to *in-vivo* methods, ideally using isolated pure carotenoids in a fat medium.

Conclusion

Identification of carotenoids requires spectral information, chromatographic properties on Tlc, HPLC and OCC and chemical tests, which must be used in conjunction for valid results. Quantification can be done by OCC and HPLC using appropriate correction factors for HPLC.

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