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A scheme for obtaining standards and HPLC quantification of leafy vegetable carotenoids

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Abstract

Reliability of data obtained by HPLC directly depends on the accuracy of the calibration. A major difficulty is obtaining and maintaining pure standards. This work presents a strategy for isolating standards by open column chromatography and quantification by HPLC, using leafy vegetables as examples. The purity of standards evaluated by HPLC was 91–97% for neoxanthin, 95–98% for violaxanthin, 97–100% for lactucaxanthin, 92–96% for lutein and 90–97% for β-carotene. Calculation procedures were also evaluated, demonstrating that results obtained with one-point recalibration, straight-line equation (until 30 days after construction of the full calibration curves) and response factors relative to β-carotene were similar (CVs of 1.6–4.0%), being well below between-sample lot natural variation (CVs of 6.1–42.5%). The scheme proposed is relatively low-cost, provides a constant supply of carotenoid standards, including those unavailable commercially, and high sample throughput.

Keywords: Carotenoids; HPLC; Analysis; Standards

1. Introduction

There is a worldwide consensus that greater and more reliable data on food carotenoids are urgently needed.

Food carotenoid analysis has been carried out to different extents, determining: (1) only the provitamin A carotenoids, (2) the principal provitamin A and non-provitamin A carotenoids and (3) the complete carotenoid composition. Since it is now recognized that carotenoids are among the phytochemicals believed to be responsible for the reduced risk of developing some degenerative diseases, this action not being linked to the provitamin A activity, the quantification of only the provitamin A carotenoids is no longer regarded as sufficient. On the other hand, determination of the complete carotenoid composition is complicated, costly and time-consuming. Considering that foods typically contain one to four or five principal carotenoids with minute or trace amounts of many other carotenoids, the added cost, time and complexity involved in identifying and quantifying minor carotenoids do not seem to be justified. Thus, the second approach appears to be the most appropriate for generating data for food data bases (Rodriguez-Amaya, 2000).

The major food carotenoids can be reliably determined either by open column chromatography (OCC) or by high performance liquid chromatography (HPLC) (Adewusi & Bradbury, 1993; Carvalho, Collins, & Rodriguez-Amaya, 1993). OCC has the advantage of using common laboratory equipment (recording UV-visible spectrophotometer) and does not require a constant supply of carotenoid standards since separated fractions are directly quantified spectrophotometrically, using published coefficients of absorption. However, the sample throughput is low and reliability of results depends heavily on the expertise of the analyst. HPLC is expensive, especially in developing countries, and reliability of results directly depends on the accuracy of the standardization. Thus, a major difficulty in HPLC analysis of carotenoids is obtaining and maintaining pure standards. The highly unsaturated carotenoids are prone to isomerization and oxidation. Although more carotenoid standards are commercially available, they are expensive especially for laboratories that have to import them. Thus, it is beneficial for a carotenoid

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laboratory to develop the expertise to isolate its own standards, including those which cannot be obtained commercially.

Beyond having good analytical methods, the organization of the execution of the analyses will determine the analytical capacity of the laboratory and will also have a direct bearing on the reliability of the results. The purpose of the present work was to establish a procedure for isolating carotenoid standards by OCC and to use these standards to determine the carotenoid composition of some leafy vegetables by HPLC as an example.

Leafy vegetables are the most accessible year-round sources of carotenoids worldwide. Leaves have a constant qualitative carotenoid pattern with lutein (β,ε,-carotene-3,3′-diol), β-carotene (β,β-carotene), violaxanthin (5,6,5′,6′-diepoxy-5,6,5′6′-tetrahydro-β,β-carotene-3,3′-diol) and neoxanthin (5′,6′-epoxy-6,7-didehydro-5,6,5′,6′-tetrahydro-β,β-carotene-3,5,3′-triol) as principal carotenoids. The quantitative composition varies considerably between leaves and reported HPLC data of the same leaf can be highly variable. In the Asian leafy vegetable *Ipomoea aquatica*, for example, Chen and Chen (1992) reported in μg/g (mean±S.D.): 100±8 β-carotene, 78±7 lutein, 60±5 violaxanthin and 50±5 neoxanthin. Wills and Rangga (1996) obtained much lower values (μg/g): 4 β-carotene, 6 lutein, 25 violaxanthin and 16 neoxanthin. Hulshof, Xu, Van de Bovenkamp and West (1997) found 27±10 μg/g (mean±S.D.) β-carotene and Tee and Lim (1991), 19 μg/g β-carotene and 34 μg/g lutein. In kale, the reported β-carotene content varied from 87 to 146 μg/g and the lutein concentration from 186 to 396 μg/g (Khachik, Beecher, & Whittaker, 1986, Micozzi, Beecher, Taylor, & Khachik 1990; Muller, 1997). For spinach, β-carotene and lutein ranged from 32 to 82 and 42 to 159 μg/g, respectively (Bureau & Bushway, 1986; Hart & Scott, 1995; Heinonen, Ollilainen, Linkola & Koivistoinen, 1989; Hulshof et al., 1997; Khachik et al., 1986; Lessin, Catigani, & Schwartz, 1997; Micozzi et al, 1990; Muller, 1997; Quackenbush, 1987; Tee & Lim, 1991). Although natural variation, due to such factors as variety or cultivar, climate, stage of maturity, may account for part of the divergence, the differences for the same foods are sometimes so wide that analytical inaccuracies appear to be involved. Thus, continued effort to evaluate and improve carotenoid methodology is warranted.

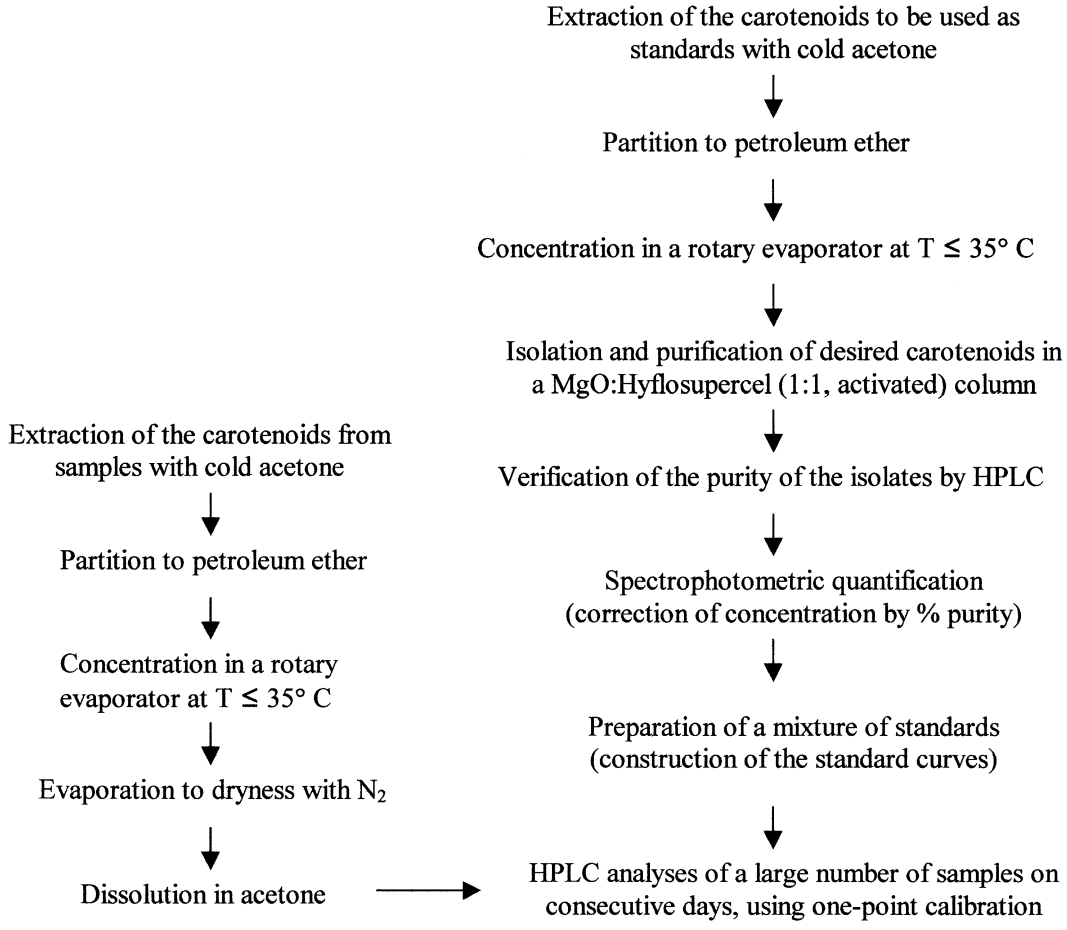

Fig. 1. Proposed scheme for obtaining standards by OCC and quantitative analysis by HPLC.

2. Materials and methods

2.1. Isolation of standards by OCC

Lettuce was used as the source of standards because it was among the samples analysed in this study, and it was the only vegetable that contained lactucaxanthin (ε, ε-carotene-3,3′-diol). Otherwise, other easy to extract green vegetables with high carotenoid content, such as cress and parsley, can be used.

Fig. 1 shows the scheme proposed. To obtain standards, the carotenoids were extracted (from about 50–60 g of curly lettuce) with cold acetone, partitioned to petroleum ether, concentrated in a rotary evaporator and separated in an open column of MgO (Merck, Germany):Hyflosupercel (1:1 activated for 2 h at 110 °C), adjusting the mobile phase, not to separate all the carotenoids present, but to isolate the desired carotenoids as quickly and efficiently as possible. The separation pattern with the eluting solvents is shown in Fig. 2. Since the objective of OCC was not quantitative analysis, only the main portion of each band of carotenoid was collected, avoiding contamination from the other bands. This was done especially with lutein and violaxanthin which eluted close to each other. Isolates eluted with petroleum ether containing acetone were washed three or four times with water in a separatory funnel to remove acetone and then dried with Na_2SO_4. A detailed description of OCC is given in Rodriguez-Amaya (1999).

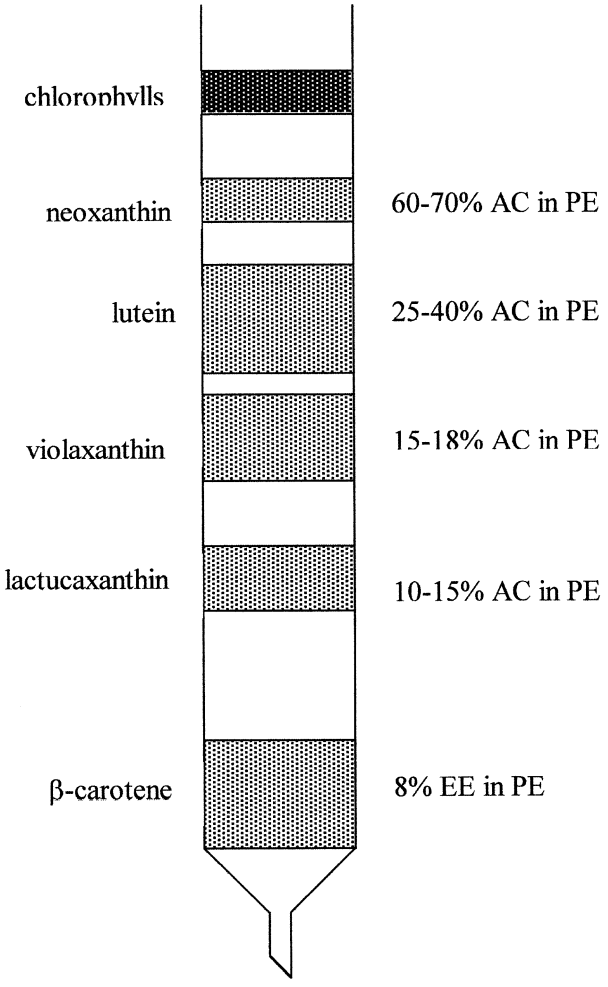

Fig. 2. Separation pattern and eluting solvents of carotenoids from unsaponifled lettuce extract on the MgO:Hyflosupercel column. EE, ethyl ether; AC, acetone; PE, petroleum ether.

The adsorption affinity of MgO can differ between brands and sometimes between batches of the same brand. MgO from Mallinckrodt (USA), for example, does not require activation. Some adjustment of the mobile phase may also be needed. In cases when separation of violaxanthin and lutein does not yield pure standards, the fraction corresponding to these carotenoids can be collected together and rechromatographed on a neutral alumina column (activity I), using 25–40% of acetone in petroleum ether to elute lutein and acetone to elute violaxanthin (Rodriguez-Amaya, 1999). We did not have to do this in our laboratory.

An aliquot was taken from each isolate to verify the purity by HPLC (i.e. a chromatogram showing a single peak corresponding to the carotenoid, giving the same characteristic spectra, obtained with a photodiode array detector, at the ascending and descending slopes and at the maximum). All aliquots were dried under N_2, and immediately before injection, dissolved in 1 ml HPLC grade acetone, filtered through a 0.22 μm PTFE syringe filter (Millipore) directly to sample vials, and 10 μl injected into the chromatograph. Once the desired purity is verified, the concentrations of the pure standards were determined spectrophotometrically, using the following $A^{1\%}_{1cm}$ values: β-carotene, 2592 in petroleum ether; lutein, 2550 in ethanol; violaxanthin, 2550 in ethanol; neoxanthin, 2243 in ethanol. For lactucaxanthin a $A^{1\%}_{1cm}$ value of 2944 in petroleum ether was calculated according to the formula which relates the absorption coefficient and the molecular masses of two carotenoids of the same chromophore (Davies, 1976), using the $A^{1\%}_{1cm}$ of 3120 of ε,ε-carotene.

2.2. Preparation of the standard solution and construction of the standard curves

Aliquots of the carotenoid isolates (in petroleum ether) were taken in volumes that would give the relative proportion found in the sample, mixed, concentrated, made up to volume, and 0.1% of butylated hydroxytoluene (BHT) was added.

For the standard curves, triplicate aliquots of 1, 2, 3, 4 and 5 ml were transferred to culture tubes, dried under N_2, and just before injection, dissolved in I ml of acetone and filtered through a 0.22 μm PTFE syringe filter (Millipore); 10 μl was automatically injected into the HPLC equipment. These curves, constructed with five

different concentrations for each carotenoid, each concentration in triplicate, should pass through or very near the origin, be linear with a correlation coefficient $\geqslant 0.95$ (Mantoura & Repeta, 1997), and should bracket the concentrations expected in the samples.

For the one-point recalibrations, aliquots of the standard mixture were quantitatively transferred into screw-capped culture tubes, dried under nitrogen, and stored at −18 °C until use.

2.3. Analysis of the leafy vegetables

Once the standard solution had been prepared and the standard curves demonstrated the required characteristics, a convenient number of samples could be analyzed each day on three to four consecutive days during the week (Fig. 1).

Three different samples of each of five vegetables (Boston lettuce, curly lettuce, roquete, cress and chicory) were analysed, one on the day the full standard curves were constructed and the other two 15 and 30 days later. The samples were analysed immediately after purchase. For each sample, the leaves from two bunches were finely cut, mixed, and 2–5 g samples were taken for analysis.

Carotenoids analysis is inherently difficult and great care is needed in carrying out this analysis. Precautionary measures to avoid artifact formation and losses of carotenoids during analysis (e.g. exclusion of oxygen, protection from light, avoiding high temperatures and contact with acids, use of high purity, peroxide-free solvents, completion of the analysis within the shortest possible time) and quality control and quality assurance measures (e.g. rigorous training of the analyst, comparison of OCC and HPLC results, evaluation of repeatability, verification of purity and stability of standards, evaluation of carotenoid recovery in the different steps) as discussed in detail in Rodriguez-Amaya (1999) were taken.

The carotenoids were extracted with cold acetone (using a mortar and pestle, which was found to be more efficient in disintegrating small amounts of leaf samples than a Waring blendor) and partitioned to petroleum ether as described for the OCC method (Rodriguez-Amaya, 1999). The extract was concentrated in a rotary evaporator ($T \leqslant 35$ °C) and dried under N_2. Immediately before injection, the residue was redissolved in 2 ml HPLC grade acetone and 1 ml was filtered with a 0.22 μm PTFE syringe filter (Millipore); 10 μl was automatically injected into the HPLC equipment. Carotenoid solutions quantified spectrophotometrically before and after filtration were found to have the same concentrations.

Identification of the carotenoids was carried out as described by Rodriguez-Amaya (1999). This involved the combined use of the retention times, co-chromatography with authentic samples, the visible absorption spectra obtained spectrophotometrically and by the photodiode array detector, and for xanthophylls, chemical tests such as acetylation with acetic anhydride of secondary hydroxy groups (as in lutein, violaxanthin, neoxanthin, and lactucaxanthin), methylation with acidic methanol of allylic secondary hydroxy groups (as in lutein and lactucaxanthin), and epoxide-furanoid rearrangement of 5,6-epoxy groups (as in violaxanthin and neoxanthin). Lactucaxanthin was first identified specifically in lettuce by Siefermann-Harms, Hertzberg, Borch, and Liaansen-Jensen (1981), the structure being elucidated by mass spectrometry and magnetic resonance spectroscopy. It is quantified for the first time in the present study. Zeaxanthin (β, β-carotene-3,3′-diol) and cis-isomers of β-carotene were also identified but were not quantified because they were present at very low levels. The β-carotene concentration reported in this paper, therefore, refers to *trans*-β-carotene.

2.4. HPLC conditions

The HPLC analysis was performed on a Waters separation module (model 2690) equipped with quaternary pump, four channel in-line vacuum degasser, and an autosampler injector, controlled by Millenium 2010 workstation, using a monomeric C_{18} column (Waters Spherisorb S3 ODS2), 3 μm, 4.6×150 mm. The mobile phase consisted of acetonitrile, methanol, and ethyl acetate containing 0.05% of TEA (triethylamine) used at a flow rate of 0.5 ml/min. The use of TEA was recommended by Hart and Scott (1995) to increase carotenoid recovery. A concave gradient (curve 10) was applied from 95:5:0 to 60:20:20 in 20 mm, maintaining this proportion until the end of the run. Re-equilibration took 15 mm. A UV-visible photodiode array detector (Waters model 996) was used. Detection was at the wavelengths of maximum absorption of the carotenoids in the mobile phase (max plot): neoxanthin, 438 nm; violaxanthin, 441 nm; lactucaxanthin, 439 nm; lutein, 447 nm, and β-carotene, 454 nm. The instrument room was maintained at 22 °C.

Metal surfaces, particularly stainless steel frits in guard and analytical columns, were reported to be damaging to carotenoids (Scott, 1992). Thus, the use of metal-free columns (e.g. with ''biocompatible'' frits) (Craft, Wise, & Soares, 1992) and poly ether ether ketone (PEEK) tubing for column connections (Hart & Scott, 1995) has been recommended. Our HPLC system was tested in terms of lycopene, the carotenoid for which high intralaboratory and interlaboratoiy coefficients of variation and low recoveries from the HPLC columns had been reported (Epler, Sander Ziegler, Wise, & Craft, 1992; Hart & Scott, 1995; Konings & Rooman, 1997; Riso & Porrini, 1997), using 10 different samples of pink-fleshed guava, a fruit in which lycopene

comprises about 93% of the total carotenoid content (Porcu & Rodriguez-Amaya, 2001). Comparison of the lycopene concentrations obtained by correcting the total carotenoid content (the total carotenoid content calculated from the spectrophotometric maximum absorbance corrected by the lycopene area relative to the total area at the wavelength of detection), by OCC and by HPLC showed no loss of lycopene in our HPLC system. Thus, the column frits and column connection tubings were not substituted.

2.5. Calculation of carotenoid concentrations

The opportunity was also taken to evaluate the procedures for the calculation of the carotenoid concentrations to verify if this step contributed to the variability of analytical data perceived from the literature. Calculation of the concentrations can be carried out in different ways, all of which attempt to compensate changes in detector's response: (a) using full standard curves constructed at each day of analysis, (b) construction of full standard curves to verify linearity over the samples' concentrations and passage through the origin and one-point recalibration on each day of analysis and (c) use of response factors. Most carotenoid papers do not specify the calculation method used. The first is the ideal procedure but it takes a long time, leaving little time for the samples on each day of analysis, thus limiting sample throughput. It also uses a lot of standards. Following gas chromatographic practices, the second procedure can be used. Injection of a standard of known concentration on each day of analysis in effect verifies any change in the slope of the standard curve (i.e. change in detector's sensitivity). Although much simpler and more rapid, it has to be done carefully because there is a danger that this single point can be an outlier. It must always be verified that this point does not deviate appreciably from the full curve. The samples' carotenoid concentrations are calculated by the formula:

$$C_x\,(\mu g/g) = \frac{A_x \times C_s\,(\mu g/ml) \times \text{total volume of extract (ml)}}{A_s \times \text{sample weight (g)}}$$

where C_x is the concentration of the carotenoid X, A_x is the peak area of the carotenoid X, C_s is the concentration of the standard and A_s is the peak area of the standard.

Use of response factors is also a simplification because a single reference carotenoid standard is injected on each day of analysis. The response factor of each carotenoid relative to the reference carotenoid is calculated by the formula (Hart and Scott, 1995):

$$RF_x = \frac{\text{peak area of carotenoid X (1 } \mu g/ml)}{\text{peak area of reference carotenoid (1 } \mu g/ml)}$$

The carotenoid concentration in the sample is calculated by the formula:

$$C_x\,(\mu g/g) = \frac{A_x \times \text{total volume of extract (ml)}}{RF_x \times A_{ref} \times \text{sample weight (g)}}$$

where C_x is the concentration of the carotenoid X, A_x is the peak area of the carotenoid X, RF_x is the response factor of the carotenoid X and A_{ref} is the peak area of 1 μg/ml of the reference carotenoid.

In the present study different leafy vegetables were quantified by HPLC using external standardization, the calculation being made by one-point recalibration, the straight line equation, and response factors relative to β-carotene and lutein.

3. Results and discussion

3.1. Purity of the isolated standards

Fig. 3 shows the HPLC chromatograms of the isolated standards. The purity calculated as the percentage of the carotenoid's peak area relative to total area was 91–97% for neoxanthin, 95–98% for violaxanthin, 97–100% for lactucaxanthin, 92–96% for lutein and 90–97% for β-carotene. The concentrations of the standards were corrected accordingly. β-carotene in leaves generally contains cis-isomers that cannot be separated in the MgO:Hyflosupercel column, decreasing the purity of the standard. This *cis*-isomers can be separated using a $Ca(OH)_2$ column (Godoy & Rodriguez-Amaya, 1994; Tavares & Rodriguez-Amaya, 1994), but this will prolong the analysis substantially. In any case, the purity percentages obtained are highly satisfactory.

Quackenbush and Smallidge (1986) evaluated the purity of commercial β-carotene and the purity by spectral absorbance ranged from 2.4 to 95.6%. Deterioration was attributed principally to autoxidation after packaging. These authors had to recrystallize the commercial β-carotene before use. Craft, Sander and Pierson (1990) found that the impurities separated by HPLC accounted for 16–75% of the absorbance of commercial β-carotene preparations at 450 nm. Based on these observations, these authors estimated that all-*trans*-β-carotene measurements could only be 1/50 of reported values.

Hakala and Heinonen (1994) isolated lycopene from tomato puree, using more sophisticated techniques: solid-phase extraction (silica cartridges) and three purifications with semipreparative HPLC. However, the purity obtained with the method developed was only 77% (20% of *cis*-isomers and 3% of xantophylls).

Fig. 4a shows a typical chromatogram of the mixture of standards. Calibration with a mixture rather than individual injection of standards saves a lot of time and the calibration chromatogram approximates that of the samples, thereby decreasing relative errors. In fact the chromatogram of the mixture of isolated standards simulates that of the leafy vegetable (Fig. 4b), without the peaks corresponding to chlorophylls. Moreover, because the standards are isolated from leafy vegetables, the concentration ratios also mimic those of the samples, making it easier to bracket the samples' concentrations.

The standard curves of each of the carotenoids passed through the origin and showed linearity, with coefficients of correlation of 0.999 for neoxanthin, violaxanthin and lactucaxanthin and 0.998 for lutein and β-carotene. The CVs (coefficients of variation) of triplicate measurements at five points of the standard curves varied from 0 to 2.0% for neoxanthin, 0.1 to 1.7% for violaxanthin, 0.1 to 1.9% for lactucaxanthin, 0 to 1.5% for lutein and 0.2 to 2.0% for β-carotene. CVs for the ratios of the concentration and the area of standard's peak corresponding to the single points for each day of analysis (15 measurements for each carotenoid over the 1-month period) were 1.1% for neoxanthin, 1.7% for violaxanthin, 0.7% for lactucaxanthin, 2.0% for lutein and 1.3% for β-carotene, well below the 5% suggested by Mantoura and Repeta (1997).

3.2. Comparison of calculation procedures

Tables 1–5 presents the carotenoid concentrations of the leafy vegetables calculated by one-point recalibration, the straight-line equation (of the standard curves constructed at the beginning of the study) and the response factors relative to β-carotene and lutein.

CVs were only 1.6–4.0%, except for β-carotene when response factor relative to lutein was used (5.0–7.5%). These CVs are much smaller than the lot-to-lot variation (6.1–42.5%).

Notably, the results obtained with the straight-line equation resembled the other results even 30 days after the construction of the full calibration curves. This procedure amounts to using the standard curves obtained on one day to quantify samples analyzed over a one-month period. This means that the detector's response of the chromatograph used did not change during this period. A significant change may occur over a longer period.

Hart and Scott (1995) used β-cryptoxanthin as the reference standard in determining carotenoid concentration, using response factors. Although these

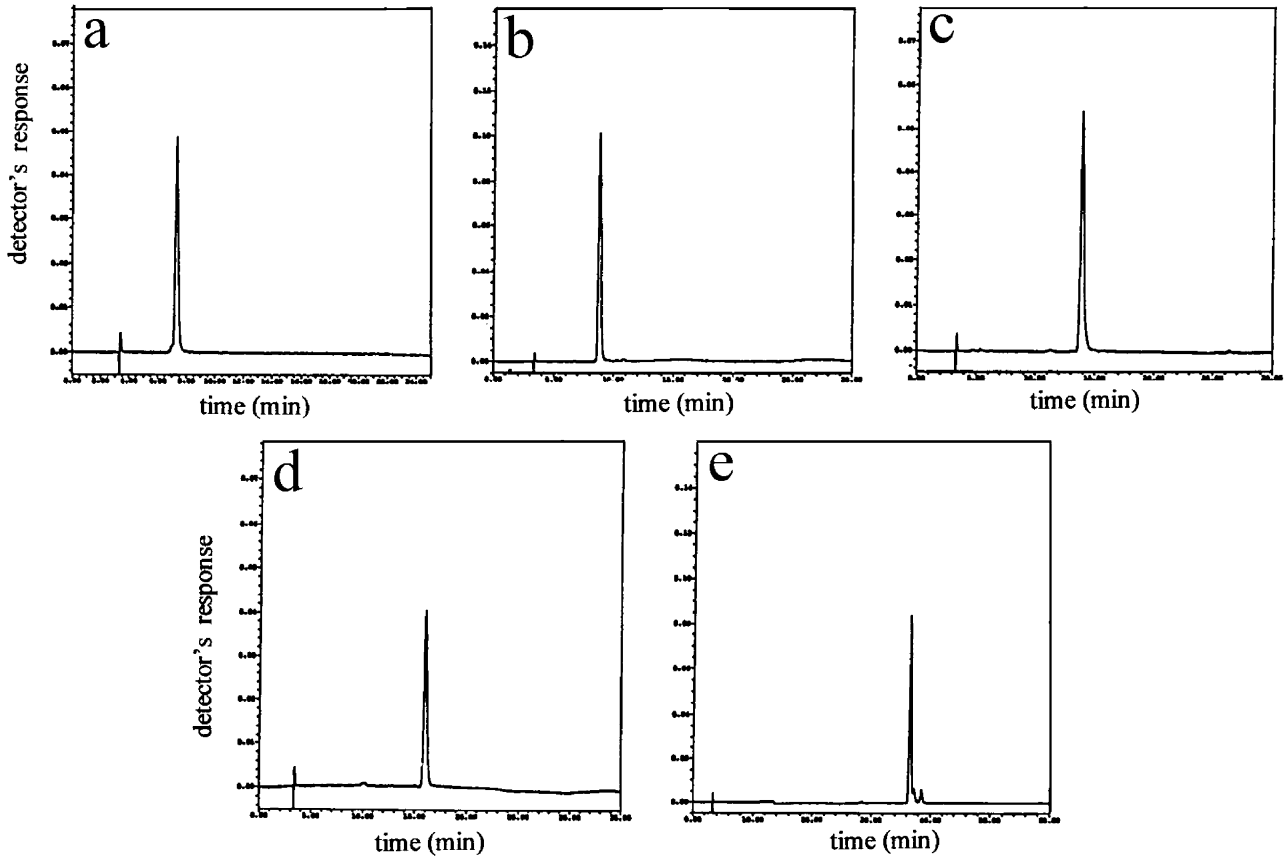

Fig. 3. HPLC chromatograms of carotenoid standards isolated from curly lettuce: (a) neoxanthin, (b) violaxanthin, (c) lactucaxanthin, (d) lutein and (e) β-carotene. HPLC conditions are described in text.

authors did not give the reason for this choice, it can be surmised that it was based on the fact the β-cryptoxanthin has an intermediate polarity, between dihydroxy xantophylls and the carotenes, thus serving as a good reference standard for carotenoids of both sides of the polarity range. β-carotene was used as reference standard in this work because it is easy to isolate and is widely available commercially at low cost. Lutein was also utilized to verify the effect of polarity. β-Carotene appeared to be an appropriate reference standard for the dihydroxy xantophylls lutein, lactucaxanthin, violaxanthin and neoxanthin. However, response factor relative to lutein appeared to be inadequate for β-carotene.

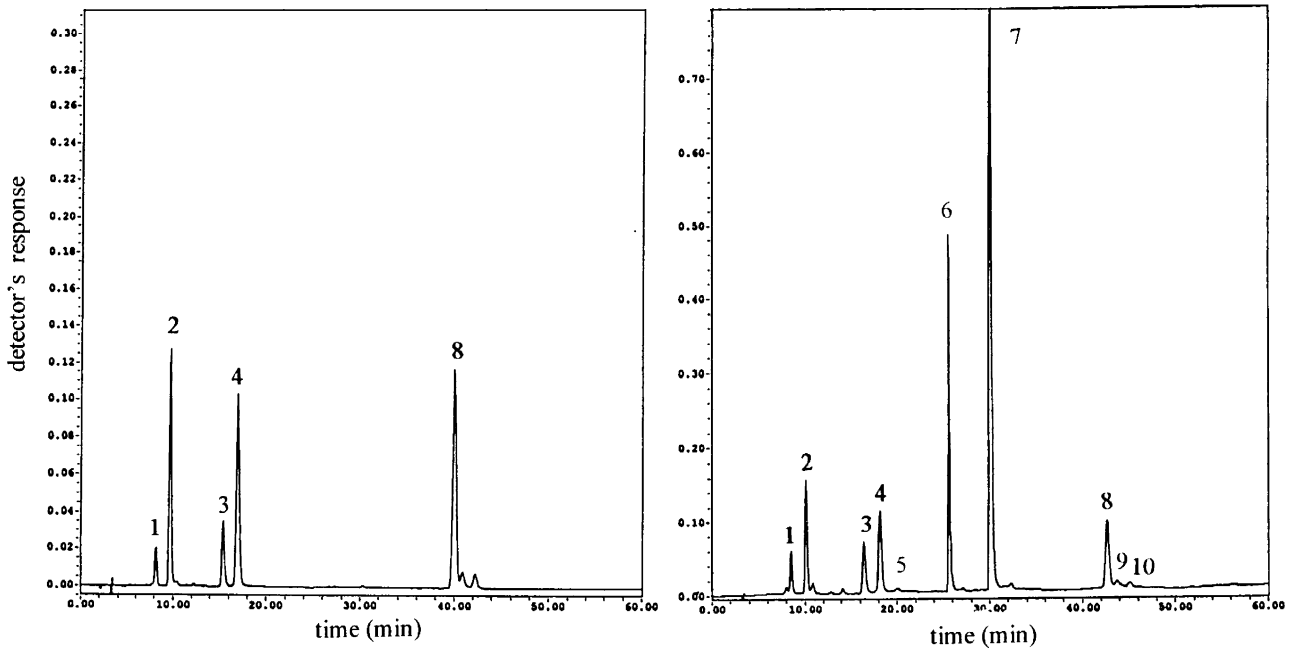

Fig. 4. HPLC chromatograms of a mixture of isolated standards (a) and extract of lettuce (b). Chromatographic conditions are described in text. Peak identification: 1, neoxanthin, 2, violaxanthin, 3, lactucaxanthin; 4, lutein, 5, zeaxanthin; 6,7, chlorophylls; 8, *trans*-β-carotene; 9,10, *cis*-β-carotene.

Table 1
Comparison of carotenoid composition (μg/g) of Boston lettuce obtained by one-point recalibration, straight line equation and response factors

Carotenoid	Sample number[a]	One-point calibration	Straight line equation	RF relative to β-carotene	RF relative to lutein	CV between calibration
Neoxanthin	1	7.0	6.7	6.9	7.3	3.0
	2	10.4	10.3	10.3	10.6	1.6
	3	11.5	11.1	10.8	11.1	2.9
CV between samples		24.3	23.9	22.3	21.3	
Violaxanthin	1	17.7	16.8	17.1	18.0	3.1
	2	17.7	17.2	17.2	17.8	1.8
	3	21.8	21.4	20.9	21.5	1.7
CV between samples		12.3	14.0	11.7	11.1	
Lactucaxanthin	1	11.7	12.0	12.0	12.8	3.9
	2	10.8	11.5	11.4	11.8	3.9
	3	12.6	13.7	13.2	13.7	3.8
CV between samples		7.9	9.4	7.5	7.3	
Lutein	1	21.0	19.5	19.7	–	4.0
	2	19.5	18.7	18.7	–	2.4
	3	23.0	22.7	22.1	–	2.1
CV between samples		8.2	10.4	8.4	–	
β-Carotene	1	22.9	22.6	–	20.8	5.0
	2	21.7	21.6	–	19.3	6.5
	3	24.5	25.2	–	21.8	7.5
Cv between samples		6.2	8.0	–	6.1	

RF = response factor; CV = coefficient of variation (%).
[a] Different samples analysed at 0 (1), 15 (2) and 30 (3) days after construction of the full standard curves.

The scheme proposed can be applied to other food samples. This implies that aside from identifying the carotenoids in the samples conclusively and defining the optimum conditions for HPLC, the procedure for isolating and purifying the carotenoids to be used as standards by OCC should be established before hand. It is not necessary that standards be isolated from the same types of food as the samples, as was done in the present work for leafy vegetables. For greater ease of isolation and to obtain greater amounts of standards, the analyst can use carotenoid-rich foods as sources of standards, such as α-carotene and β-carotene from carrots, β-cryptoxanthin from papaya and lycopene from tomato.

For greater efficiency, food samples to be analyzed should be grouped according to the carotenoid composition, and samples of similar composition should be analyzed together so that the same standard solution can be used and the greatest number of samples can be

Table 2
Comparison of carotenoid composition (μg/g) of curly lettuce obtained by one-point recalibration, straight line equation and response factors

Carotenoid	Sample number[a]	One-point calibration	Straight line equation	RF relative to β-carotene	RF relative to lutein	CV between calibration
Neoxanthin	1	5.6	5.4	5.4	5.6	2.2
	2	6.7	6.6	6.6	6.9	1.6
	3	9.0	8.7	8.4	8.7	2.9
CV between samples		24.7	24.0	22.6	22.3	
Violaxanthin	1	15.2	14.2	14.1	14.7	3.5
	2	14.8	14.4	14.5	14.9	1.8
	3	16.7	16.4	16.0	16.5	1.7
CV between samples		6.2	8.1	6.4	6.7	
Lactucaxanthin	1	8.7	9.0	8.9	9.3	2.7
	2	7.6	8.1	8.0	8.4	3.9
	3	9.5	10.3	9.9	10.3	3.8
CV between samples		11.0	12.0	10.4	10.4	
Lutein	1	15.6	15.0	14.9	–	2.6
	2	15.4	14.8	14.8	–	2.4
	3	17.9	17.7	17.2	–	2.1
CV between samples		8.4	10.2	8.6	–	
β-Carotene	1	16.9	17.0	–	15.2	6.4
	2	18.2	18.1	–	16.2	6.5
	3	19.4	20.0	–	17.3	7.5
CV between samples		7.0	8.1	–	6.6	

RF = response factor; CV = coefficient of variation (%).
[a] Different samples analysed at 0 (1), 15 (2) and 30 (3) days after construction of the full standard curves.

Table 3
Comparison of carotenoid composition (μg/g) of roquette obtained by one-point recalibration, straight line equation and response factors

Carotenoid	Sample number[a]	One-point calibration	Straight line equation	RF relative to β-carotene	RF relative to lutein	CV between calibration
Neoxanthin	1	9.5	9.2	9.1	9.5	2.1
	2	8.1	7.7	7.4	8.0	3.6
	3	13.8	13.3	12.9	13.3	2.8
CV between samples		28.5	28.5	28.4	27.0	
Violaxanthin	1	20.9	19.4	19.4	20.1	1.7
	2	12.0	12.2	11.8	12.6	2.8
	3	28.3	27.9	27.2	28.0	1.7
CV between samples		40.1	39.4	39.5	38.1	
Lutein	1	49.7	47.7	47.3	–	2.6
	2	33.0	31.9	30.6	–	3.7
	3	67.4	66.6	64.7	–	2.1
CV between samples		34.3	35.6	35.8	–	
β-Carotene	1	32.7	32.9	–	29.3	6.3
	2	19.2	19.9	–	17.6	6.1
	3	47.3	48.6	–	42.1	7.5
CV between samples		42.5	42.5	–	41.2	

RF = response factor; CV = coefficient of variation (%).
[a] Different samples analysed at 0 (1), 15 (2) and 30 (3) days after construction of the full standard curves.

analyzed. The scheme proposed projects a 1-week activity. The standards can be isolated and purified, their purity checked, and the standard mixture solution can be prepared on the first day. The standard curves can be made on the second day. Extraction and HPLC analyses of a large number of samples can then be carried out in the next consecutive days. The limiting factor will be the time for each HPLC run. In the following weeks, one-point recalibration can be employed throughout, increasing the number of days of analysis (about 8 days) and sample throughput, provided these points fall close to the curves. Mantoura and Repeta (1997) recommended that full calibration be done every 3–4 months or when variation of the ratio between concentration and area of standard's peak exceeds 5%.

In subsequent evaluation, the standards were found to be stable in 15 days, provided that the culture tubes were kept in a vacuum desiccator. It is also possible to extract a greater amount of standards and the aliquots with BHT stored in sealed vials under N_2, at the lowest temperature possible ($< -18\,^{\circ}C$), for use over an extended period.

Table 4
Comparison of carotenoid composition (μg/g) of cress obtained by one-point recalibration, straight line equation and response factors

Carotenoid	Sample number[a]	One-point calibration	Straight line equation	RF relative to β-carotene	RF relative to lutein	CV between calibration
Neoxanthin	1	14.4	13.9	13.8	14.4	2.1
	2	13.1	12.9	12.9	13.4	1.6
	3	20.1	19.3	18.8	19.4	2.8
CV between samples		23.6	22.4	20.8	20.6	
Violaxanthin	1	20.9	19.4	19.3	20.1	3.5
	2	23.6	22.9	23.0	23.7	1.8
	3	27.7	27.3	26.6	27.4	1.7
CV between samples		14.3	16.9	15.7	15.4	
Lutein	1	61.4	59.0	58.5	–	2.6
	2	77.8	74.6	74.6	–	2.4
	3	80.7	79.8	77.4	–	2.1
CV between samples		14.2	15.2	14.6	–	
β-Carotene	1	28.4	28.6	–	25.5	6.3
	2	40.5	40.4	–	36.1	6.5
	3	39.4	40.5	–	35.1	7.5
CV between samples		18.5	18.7	–	18.1	

RF = response factor; CV = coefficient of variation (%).
[a] Different samples analysed at 0 (1), 15 (2) and 30 (3) days after construction of the full standard curves.

Table 5
Comparison of carotenoid composition (μg/g) of chicory obtained by one-point recalibration, straight line equation and response factors

Carotenoid	Sample number[a]	One-point calibration	Straight line equation	RF relative to β-carotene	RF relative to lutein	CV between calibration
Neoxanthin	1	9.1	8.9	8.8	9.2	2.1
	2	18.2	18.0	17.3	18.5	3.6
	3	18.4	17.7	17.2	17.8	2.8
CV between samples		35.3	35.0	35.0	34.3	
Violaxanthin	1	15.4	14.4	14.3	14.9	3.5
	2	23.4	23.8	23.0	24.5	2.8
	3	24.9	24.5	23.9	24.6	1.7
CV between samples		23.9	27.1	25.9	26.2	
Lutein	1	41.4	39.8	39.5	–	2.6
	2	69.0	66.7	64.0	–	3.7
	3	61.6	60.9	59.1	–	2.1
CV between samples		24.9	25.3	24.0	–	
β-Carotene	1	24.9	25.1	–	22.4	6.3
	2	43.1	44.8	–	39.7	6.1
	3	40.9	42.0	–	36.4	7.5
CV between samples		27.3	28.6	–	32.8	

RF = response factor; CV = coefficient of variation (%).
[a] Different samples analysed at 0 (1), 15 (2) and 30 (3) days after construction of the full standard curves.

The strategy herein described is low-cost and provides a constant supply of carotenoid standards, including those which cannot be acquired commercially. Sample throughput is high.

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