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Comparison of 3 Spectrophotometric Methods for Carotenoid Determination in Frequently Consumed Fruits and Vegetables

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ABSTRACT: Carotenoids are C-40 tetraterpenoid compounds with potential health beneficial effects. Major dietary sources include a variety of fruits and vegetables. Rapid screening methods are therefore desired, but their accuracy varies depending on the carotenoid profile and the matrix of the plant food. In the present study, 3 different methods were compared, all based on a rapid extraction protocol and spectrophotometric measurements to determine the total amount carotenoids present in fruits and vegetables ($n = 28$), either with or without chlorophyll. Published methods (a) Lichtenthaler and (b) Hornero-Méndez and Mínguez-Mosquera were compared with a newly developed method (method c) based on the average molar absorption coefficient (135310 $Lcm^{-1}mol^{-1}$) and wavelength (450 nm in acetone), for the 5 predominant carotenoid species (beta-carotene, zeaxanthin, lycopene, lutein, beta-cryptoxanthin) in the investigated foods. All results were compared to HPLC (method d). To avoid overestimating carotenoid concentrations due to chlorophyll A and B presence, the effect of saponification was studied for all methods. Overall, saponification led to significant carotenoid losses (12.6 ± 0.9%). Methods a, b, c, and d yielded 5.1 ± 0.4 mg/100 g, 4.6 ± 0.5 mg/100 g, 4.3 ± 0.5 mg/100 g, and 4.2 ± 0.5 mg/100 g total carotenoids, respectively, with method a leading to significant higher mean concentrations compared to all other methods ($P < 0.001$, Bonferroni) with methods b and c being not significantly different and highly correlated compared to HPLC ($> r = 0.95$). Similar results were found when stratifying for chlorophyll content and fruits compared with vegetables, however, accuracy varied for individual fruits, highlighting the limitation to use the same method for all plant foods.

Practical Application: This study presents a comparison of various rapid spectrophotometric measurements to determine total carotenoid content in various fruits and vegetables and could aid in the selection of the appropriate method for individual plant foods with different carotenoid profile and matrices.

Keywords: carotenoids, HPLC, plant foods, screening, spectrophotometry

Introduction

Carotenoids constitute a pigment family comprising more than 700 different species, consisting of a C-40 polyene backbone with conjugated double bonds. Modifications at one or both ends of the structure, that is, cyclization or the introduction of oxygen functions yield different species. The presence of the latter functions establishes the basis for a general classification into oxygen containing (xanthophylls) and nonoxygen containing carotenoids (carotenes) (Britton 1995). Besides the well-known provitamin A activity of some carotenoids, further potential health beneficial properties have moved into the focus of investigation, especially those related to the prevention of a number of chronic diseases. For example, there is evidence for a protective effect of lutein and zeaxanthin on the eye from photooxidation (Landrum and others 1997; Bernstein and others 2001). Furthermore, an inverse correlation of high carotenoid intake derived from plant foods and a reduced incidence of several types of cancer was proposed, that is, for lung cancer (Smith 1998; Wright and others 2003) and prostate cancer (DePrimo and others 2001; Giovannucci and others 2002). Results from epidemiological studies further indicate that carotenoid concentration in plasma seems to be directly associated with the mortality rate in the elderly (Lauretani and others 2008; Akbaraly and others 2009).

Carotenoids are synthesized by all plants and many microorganisms (that is, bacteria and fungi), but not by animals, including humans, who therefore rely on dietary uptake. Main dietary sources include colored vegetables and fruits (Müller 1997), but also eggs (Schlatterer and Breithaupt 2006), and some fish (Ytrestoyl and others 2004). Due to the relation of carotenoid intake and chronic diseases, methods allowing for the rapid, accurate determination of carotenoids in these matrices are highly desired.

The classical method to measure carotenoids is via basic spectrophotometric methods (Schertz 1923; Schon 1935; Gillam and Heilbron 1936; Peng and others 2005; Davey and others 2006; Bunea and others 2007; Kimura and others 2007); however, without prior separation carotenoids will be determined together with chlorophylls, absorbing at similar wavelengths. Today, carotenoids are often measured by reverse-phase high-performance liquid chromatography (RP-HPLC), allowing for individual carotenoid detection and quantification (Ferruzzi and others 1998; Unlu and others 2005), with the disadvantage of being more time and cost intensive. Still, classical spectrophotometric measurements are widely used and have been adapted for special purposes, that is, the analysis of food items rich in specific carotenoid species employing selected wavelengths and their corresponding absorption coefficient, for

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example, for red pepper extracts (Hornero-Méndez and Mínguez-Mosquera 2001). Such methods may be more accurate for special food items, but often are limited in the quantification of a broad variety of food items. On the other hand, most plant foods contain predominantly beta-carotene, lycopene, zeaxanthin, lutein, and beta-cryptoxanthin and an average absorption coefficient may be established for spectrophotometric methods. However, to overcome the problem of carotenoid overestimation by the presence of chlorophyll, a saponification step is often included during extraction, although leading to carotenoid losses (Lietz and Henry 1997). Another strategy is based on the mathematical estimation of the chlorophyll content within a carotenoid-containing solution (Lichtenthaler 1987), determining chlorophyll concentrations at wavelengths where carotenoids do not absorb. This method was created for the parallel quantification of chlorophylls and carotenoids in green leaves or separated chloroplasts.

In the present investigation, we compared 3 spectrophotometric methods (Hornero-Méndez and Mínguez-Mosquera, Lichtenthaler, and an in-house method based on mean absorption coefficient and wavelength) for their total carotenoid concentration within different chlorophyll and nonchlorophyll containing matrices. A total of 28 different fruits and vegetables were investigated to assure accuracy of results over a wide selection of plant foods, combining a fast liquid–liquid extraction preceded by a saponification step and spectrophotometric measurements. For validation, results were additionally analyzed by an HPLC protocol adapted from Gorocica-Buenfil and others (2007).

Materials and Methods

Chemicals

All products were of analytical grade or higher. Acetone was obtained from Merck (Darmstadt, Germany); ethanol, methanol, and ammonium acetate from BioSolve (Valkerswaard, the Netherlands); hexane and $CaCO_3$ from VWR (Haarode, Belgium); methyl-tert-butyl ether (MTBE) from Sigma-Aldrich (Bornem, Belgium). Water was prepared with a purification system from Millipore (Brussels, Belgium).

Carotenoid standards were purchased from 3 different suppliers: capsanthin and beta-cryptoxanthin from Extrasynthèse (Lyon, France); beta-carotene, neoxanthin, violaxanthin from CaroteNature (Bern, Switzerland); lutein, lycopene, and zeaxanthin from Sigma-Aldrich.

Selection of fruit and vegetable samples

Twenty-eight different fruits and vegetables (Table 2) were bought at the main local grocery chain (CACTUS S.A., Esch-sur-Alzette, Luxembourg) in June 2008. Samples were chosen according to carotenoid content (>0.2 mg/100 g, Souci and others 2000) and food disappearance data from Luxembourg, supplied by CACTUS S.A. headquarters (Windhof, Luxembourg). Five samples of each species were chopped into cubes of approximately 0.5 cm^2, pooled, and 5 to 15 g were weighed into 15-mL centrifuge tubes and stored at −25 °C under a blanket of argon until analysis.

Carotenoid extraction procedure

All procedures were carried out on ice and under dim light as much as possible. For extraction and saponification, a protocol was adapted from a method described earlier (Gorocica-Buenfil and others 2007). In short, weighed samples (5 to 15 g) were thawed and an equal weight of sand to facilitate homogenization, plus 1 g of calcium carbonate to neutralize cytosolic acids were added, mixed, and homogenized with a mortar and ground under liquid nitrogen. An aliquot of 2 g was weighed into a 15-mL centrifuge tube (BD Biosciences, San Jose, Calif., U.S.A.) and 5 mL of methanol were added. Additionally, 1 mL of 30% methanolic potassium hydroxide (KOH) was added. After vortexing (1 min), mixing, and incubation for 15 min on ice, samples were centrifuged (Harrier 18/80 refrigerated centrifuge, MSE, U.K.) for 5 min at 2500 × g at room temperature. A preliminary study (results unpublished) indicated that a 15-min incubation time was sufficient to remove chlorophylls, and this incubation time was consequently used for the different foods. The supernatant was decanted into a 50-mL centrifuge tube, extraction was repeated twice with 8 mL of a mixture of hexane : acetone (1 : 1, v : v) and organic fractions were combined. To the combined extracts, 25 mL of saturated aqueous sodium chloride solution was added and the mixture shaken. The supernatant hexane phase was transferred into a 50-mL centrifuge tube, and the lower aqueous phase was reextracted with 8 mL of hexane and combined with the 1st extract. Hexane extracts were weighed exactly for volume determination. A 5-mL aliquot was then pipetted from the combined extracts into a 12-mL glass vial, evaporated to dryness under a stream of nitrogen in a TurboVapLV® apparatus (Caliper Life Sciences Benelux, Teralfene, Belgium), covered with argon, and sealed. All samples were stored at −25 °C until HPLC analysis. For spectrophotometric measurements, an additional 1-mL aliquot was evaporated, redissolved in acetone, sonicated, and measured directly.

A similar extraction protocol was carried out for nonsaponified food samples, except for the addition of methanolic KOH, which was replaced with 6 mL of methanol followed by proceeding with centrifugation.

Spectrophotometric analyses

Dried extracts were reconstituted in 1 to 10 mL of acetone and sonicated for 2 min. Visible spectra (340 to 700 nm, 1 nm interval) were collected using a 1-mL quartz cuvette (101-QS, Hellma GmbH, Müllheim, Germany) in a DU 800 UV/Visible spectrophotometer (Beckman Coulter, Palo Alto, Calif., U.S.A.). For some samples (that is, blackberries), a filtration step with 0.22 μm GHP membrane filter (Pall Life Science, Ann Arbor, Mich., U.S.A.) had to be included to reduce turbidity.

According to the Beer–Lambert law, the absorbance of a dissolved, pure compound is linked to its concentration:

$$A = \varepsilon_\lambda * c * d \quad (1)$$

where A is the measured absorbance, ε_λ the molar absorption coefficient ($Lmol^{-1}cm^{-1}$) of the compound at specific wavelength λ, obtained from Rodriguez-Amaya and Kimura (2004), c being the molar concentration (mol/L) of the compound, and d the width of the cuvette (usually 1 cm). However, this formula applies only for a single species in solution; total absorbance at a given wavelength in a complex solution is equal to the sum of absorbances of each single compound. Absorption spectra of extracts from fruits or vegetables, containing chlorophylls and carotenoids, are therefore difficult to analyze as the wavelength of absorbance for both chlorophylls and carotenoids partly overlap. Depending slightly on the solvent, chlorophyll A and B possess 2 major absorbance peaks at 650 and 660 nm and between 400 and 440 nm, where carotenoids also show a characteristic absorption of typically 3 peaks or shoulders (Figure 2). Therefore, the absorbance (and the concentration) of carotenoids is overestimated in a solution containing chlorophylls. To solve this problem, several methods have been developed and compared in this investigation.

Method of Lichtenthaler (method a). According to Lichtenthaler (1987), total carotenoids can be determined after having subtracted the concentration of chlorophyll A and B, using wavelengths 661.6 and 644.8 nm, respectively, and corresponding absorption coefficients at which carotenoids do not absorb.

Method according to Hornero-Méndez and Mínguez-Mosquera (method b). According to the authors, total carotenoid content in paprika and red pepper oleoresins can be divided into red and yellow isochromic fractions. The red fraction is mainly composed of capsanthin and capsorubin whereas the yellow fraction contains, among others, zeaxanthin and beta-carotene. Absorbance values and corresponding absorption coefficients at 472 (yellow) and 508 (red) nm are used due to highest specificity of the 2 isochromic fractions at these wavelengths. Although this method was specifically developed for determining carotenoids from *Capsicum annuum*, we concluded in a preliminary study that this method could also be reliable for determining carotenoid concentrations of other vegetables or fruits, after removing potentially interfering chlorophylls.

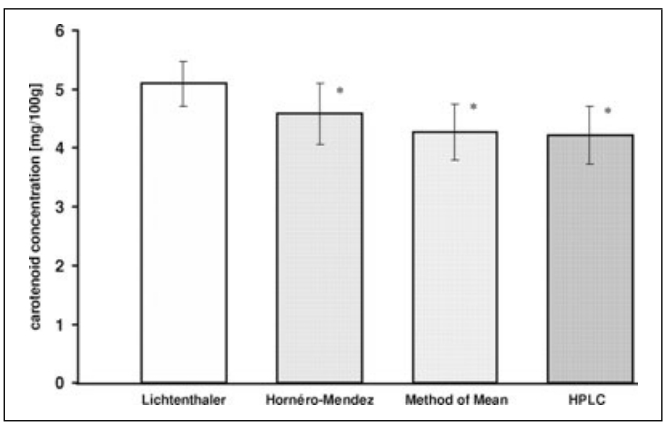

Figure 1—Mean total carotenoid concentration assessed by methods according to Lichtenthaler (1987), Hornero-Méndez and Mínguez-Mosquera (2001), Method of Mean (in-house), and HPLC (Gorocica-Buenfil and others 2007) of $n = 28$ different fruits and vegetables. Means and standard deviations were calculated as an average from each single mean and each standard deviation of the individual food items (Table 2). *Indicates statistical significant difference, $P < 0.001$ (Bonferroni).

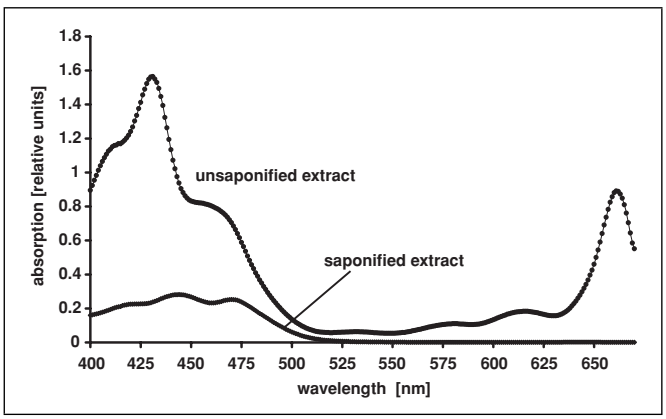

Figure 2—Effect of saponification on carotenoid extracts obtained from leek. The typical chlorophyll interference between 400 to 480 nm and consequent overestimation of the carotenoid concentration was avoided due to treatment with 30% methanolic KOH for 15 min prior to pigment extraction.

Method based on the mean absorption coefficients and mean absorption wavelength ("Method of Mean," method c). Close to 90% of the carotenoids in the diet and human body are represented by beta-carotene, alpha-carotene, lycopene, lutein, and cryptoxanthin (Rao and Rao 2007). Taking into account solely these major abundant carotenoids and exchanging alpha-carotene to zeaxanthin for a broader carotenoid spectrum, an average molar absorption coefficient and absorption wavelength can be obtained (Table 1) for carotenoid quantification, especially given that absorption maxima typically do not vary more than 5 to 10 nm. To calculate the average carotenoid concentrations (mol/L), the following equation was used:

$$c(mol/L) = \frac{A_{450} * Fd}{135310} \quad (\mathrm{d} = 1\,\mathrm{cm}) \tag{2}$$

with A_{450} being the mean absorbance maximum (A_{450}), and F a dilution factor adjusting for extractions, drying, and reconstitution processes. Using an average molar mass (g/mol), results can also be expressed as gram per liter (g/L) and as milligrams per 100 grams of edible portion (mg/100 g).

Effect of saponification

For the estimation of carotenoid losses due to saponification, Lichtenthaler and HPLC ($n = 28$ plant foods) were chosen, as both carotenoid quantification methods should be resistant to the presence of chlorophyll. Therefore, both methods were conducted with and without saponification and results compared.

HPLC analysis

Carotenoids were separated and identified by a Dionex HPLC instrument including a P580 pump and Gina 50 autosampler in combination with a UVD340S photodiode array detector (Dionex Benelux B.V., Amsterdam), by their retention times and spectral data as compared to individual standards. For quantification, 10-point calibration curves based on external standard solutions were obtained. The detector was simultaneously set at 450 (detection of alpha- and beta-carotene, beta-cryptoxanthin, lutein, and zeaxanthin) and 472 nm (detection of capsanthin, neoxanthin, violaxanthin, and lycopene).

The separation protocol was slightly adapted from a previous one (Gorocica-Buenfil and others 2007). In short, a guard column (C30, 4.0 × 20 mm) protected a YMC reverse phase C30 column (Waters Inc., 150 × 4.6 mm, 3 μm particle size, set at 28 °C), and used in combination with a binary gradient consisting of solvent (A) methanol/water/ammonium acetate/MTBE (88 : 5 : 2 : 5 by volume) and solvent (B) MTBE/methanol/water/ammonium acetate (79 : 16 : 3 : 2 by volume). The gradient started at 100% A (0 to 5 min). Solvent B was increased to 65% (during min 5 to 26) and further increased to 100% until min 34, holding this constant for 5 min. The gradient was changed back to 100% solvent A until min 40, keeping this constant for 4 min. The injection volume was 25 μL and the flow rate was kept constant at 1.2 mL/min.

Statistical analysis

For individual plant foods, carotenoid analyses were performed at least in triplicate ($n = 3$ to 10). Data were examined with SPSS 16.0 for Windows (SPSS Inc., Chicago, Ill., U.S.A.). Carotenoid concentrations were log-transformed to achieve a normal distribution. A linear mixed model was created with log-carotenoid concentration as the dependent variable, and food item and method (Lichtenthaler, Hornero-Mendez, Method of Means and HPLC) as the independent variables. In a 2nd linear mixed model, the effect of saponification was investigated separately, with

log-carotenoid concentration as the dependent variable and food item, saponification (yes, no), and method (Lichtenthaler, HPLC) as the independent variables. Pearson correlation coefficients were calculated for the correlation between the different spectrophotometric methods and HPLC. *P*-values < 0.05 were considered statistically significant different (2-sided). Where needed, Fisher *F*-tests were followed by post hoc tests (Bonferroni).

Results

Repeatability, reproducibility and limit of detection/quantification

The repeatability was assessed by comparing apricot samples on a single day in octuplicate. The spectrophotometric methods showed a similar relative standard deviation (RSD) of 7.0%, 6.9%, 6.9%, and 6.1%, for methods a, b, c, and d, respectively. The reproducibility was estimated at 5.4%, 5.7%, and 7.0% for the 3 spectrometric methods, based on triplicate analysis conducted on 3 different days. Limit of detection (LOD) for the methods was assessed as 51 μg/100 g (average of the 3 methods) and 102 μg/100 g as limit of quantification (LOQ), based on independently measured spinach samples, following the method proposed by the U.S. Environmental Protection Agency (Zorn and others 1997). In short, a diluted Arugula extract was measured 7 times and mean carotenoid concentration ± SD was determined, yielding the LOD (LOD = 3 × SD) and the LOQ (LOQ = 6 × SD).

Carotenoid losses due to saponification

The addition of methanolic KOH during extraction allowed for the degradation of chlorophyll, which could no more be detected during spectrophotometric analysis (Figure 2). Without saponification, the Method of Mean and Hornero-Méndez and Mínguez-Mosquera yielded significantly higher results (6.0 ± 0.4 and 5.8 ± 0.4 mg/100 g, respectively) compared to HPLC (4.2 ± 0.5 mg/100 g, Bonferroni, $P < 0.001$) due to chlorophyll interference. The average carotenoid losses due to saponification on the other hand were 12.6 ± 0.9% (range 0% to 58%, for banana and endive, respectively) when comparing results obtained by Lichtenthaler and HPLC from unsaponified ($n = 28$) and saponified samples ($n = 28$; $P < 0.001$, Fisher *F*-test) with both methods resulting in similar losses. When stratifying for chlorophyll ($n = 14$) compared with nonchlorophyll containing ($n = 14$) plant foods, carotenoid losses were 16.6 ± 0.9% compared with 7.3 ± 0.8%, respectively (Figure 3).

Comparison of methods

For the comparison of the various methods, data obtained by HPLC (unsaponified), Lichtenthaler (unsaponified), Hornero-Méndez (saponified), and the Method of Mean (saponified) were examined (Table 2), with the 1st 2 methods in theory being invariant to the presence of chlorophyll. The Lichtenthaler method resulted on average in 15% higher (5.1 ± 0.4 mg/100 g) carotenoid concentrations compared to the other 3 methods ($P < 0.001$, Bonferroni), while HPLC showed lowest concentrations (4.2 ± 0.5 mg/100 g), with Method of Mean (4.3 ± 0.5 mg/100 g) and Hornero-Méndez and Mínguez-Mosquera (4.6 ± 0.5 mg/100 g) being in between (Table 2), but not significantly different from HPLC (Figure 1). The results of all 3 spectrophotometric methods were positively and significantly correlated with HPLC, with the methods described by Lichtenthaler and by Hornero-Méndez and Mínguez-Mosquera showing lowest (both $r = 0.95$; $P < 0.001$) and the Method of Mean showing highest correlation coefficients ($r = 0.96$; $P < 0.001$). Stratifying for chlorophyll-containing plant food, similar results were obtained. Considering only the nonchlorophyll containing foods, Lichtenthaler yielded highest results compared to the other methods ($P < 0.001$, Bonferroni), followed by Hornero-Méndez and Mínguez-Mosquera and Method of Mean. HPLC resulted in significantly lower values than Hornero-Méndez and Mínguez-Mosquera ($P < 0.01$, Bonferroni). Comparing fruits and vegetables also led to similar results: Lichtenthaler method yielded highest values while the other 3 methods were significantly lower ($P < 0.001$, Bonferroni), but not different from each other. As methods were originally developed for different plant foods, larger variations were noted for some specific food items. For example, spectrophotometric results for watermelon were, on average, 3.5-fold higher than HPLC results. Method of Mean yielded closest results for lycopene-rich foods. However, carotenoid contents were overestimated 1.1-fold in tomatoes and 2.7-fold in watermelon (Table 2).

Comparison of food items (HPLC)

Carrots showed highest carotenoid concentrations of the 28 foods investigated (18.0 ± 2.1 mg/100 g), followed by red bell pepper (15.9 ± 1.9 mg/100 g) and spinach (12.7 ± 1.7 mg/100 g). In apple (0.20 ± 0.01 mg/100 g), broccoli (0.20 ± 0.01 mg/100 g), and grape (0.11 ± 0.02 mg/100 g), lowest carotenoid concentrations were found.

Discussion

Carotenoids have moved into the focus of attention due to the association of dietary intake and reduced incidence of a number of chronic diseases. Carotenoids can be found in a variety of food sources, especially in green-leafy vegetables and colored fruits, which can be considered as the major sources for carotenoids (Mangels and others 1993). Thus, determination of

Table 1 — Absorption coefficients (ε) of major carotenoids present in frequently consumed fruits and vegetables. Coefficients are given for their maximal absorbance wavelength (nm) and corrected by own spectrophotometric data for acetone if the solvent in literature was different.

Compound	Solvent	λ_{max} [nm]*	λ_{ad} [nm]	ε [L/mol]*	*m* [g/mol]
β-Carotene	Acetone	452	452	140663	537
β-Cryptoxanthin[a]	Petrol ether	449	453	131915	553
Lutein	Ethanol	445	448	144900	545
Lycopene[b]	Acetone	448	448	120600	537
Zeaxanthin	Acetone	452	452	133118	569
	Mean	449	450.2	135310	548
	SD	2.7	1.9	7979	14
	RSD [%]	0.6	0.5	5.9	2.6

*Rodriguez-Amaya and Kimura 2004.
[a]Beta-cryptoxanthin was used for total cryptoxanthin due to high similarity between the alpha and beta isomer.
[b]Absorption coefficient and wavelength taken from Budavari (1989).
λ_{max} = wavelength at maximum absorption.
λ_{ad} = adapted wavelength for acetone.

carotenoid intake, together with their measurement in food matrices, is an important tool for assessing dietary qualities. While HPLC methods allow for detailed carotenoid separation and quantification, but are relatively time and cost intensive, simple spectrophotometric methods are still widely used for various purposes and food matrices (Lichtenthaler 1987; Hornero-Méndez and Mínguez-Mosquera 2001; Peng and others 2005; Davey and others 2006; Bunea and others 2007; Kimura and others 2007). However, many of these methods were often not compared to other spectrophotometric methods and not always validated by HPLC, or have been used only for a limited variety of plant foods or specific carotenoid compounds.

In the present investigation, we have compared 2 published and a modified in-house spectrophotometric method to HPLC, for the rapid quantification of total carotenoids in a broad variety of matrices. Compared to results obtained by HPLC, which served as the present standard method and was based on a previously published report (Gorocica-Buenfil and others 2007), the method according to Hornero-Méndez and Mínguez-Mosquera and the Method of Mean, delivered most similar, not significantly different results for various carotenoid containing matrices overall, being on average 7.4% and 1.2% higher than HPLC results, with good correlation to the latter, suggesting both methods could be used for screening purposes for the majority of plant foods investigated. Using the Lichtenthaler method, which does not rely on a saponification step as the chlorophyll content is subtracted mathematically, the average carotenoid concentration was found to be significantly higher than results derived from all other methods, by about 21% as compared to HPLC, with stronger carotenoid overestimation of chlorophyll-rich foods, perhaps due to mathematical underestimation of the absorption contribution by the chlorophyll fraction. Otherwise, similar performance of methods was obtained when stratifying for chlorophyll compared with nonchlorophyll containing foods or when contrasting for fruits and vegetables.

However, also the Hornero-Méndez and Mínguez-Mosquera and the in-house method over- and underestimated carotenoid content of some individual plant foods, by up to 71% (spinach) and 35% (banana) compared to HPLC, respectively. In the case of watermelon, carotenoid concentration was overestimated 2.7- to 4.1-fold by all the 3 spectrophotometric methods. It has already been reported earlier that spectrophotometric methods tend to overestimate carotenoid content when compared to HPLC due to other compounds also detected, for example, carotenoid degradation products (Kimura and others 2007) and chlorophyll degradation products, that is, chlorophyllides (Almela and others 2000) also absorbing at similar wavelengths, even though their increased polarity due to phytol cleavage would result only in small extractability in hexane (Chiba and others 1967). Lower carotenoid concentrations as opposed to HPLC were less frequently measured, and may be explained by saponification losses. Carotenoid losses have been reported as the relatively harsh conditions of alkaline treatment resulted in the destruction of carotenoids (Khachik and others 1986; Oliver and others 1998; Rodriguez-Amaya and Kimura 2004) and the polyene backbone, resulting in altered light absorption patterns. In the present study, average carotenoid losses of about 12.5% due to saponification were detected, while only about 8% losses were detected in nonchlorophyll containing foods, hypothetically due to different carotenoid distribution in the plant matrix or varying absolute carotenoid concentrations.

However, the individual methods resulted in varying accuracy for different plant foods. The Lichtenthaler method tended to overestimate the carotenoid concentration in tomato, as total carotenoid absorption is measured at a wavelength close to lycopene absorption maximum (471 nm), with lycopene possessing a higher molecular absorption coefficient as compared to the average carotenoid mixture usually present in fruits and vegetables for which the Lichtenthaler method was developed for (470 nm, $A^{1\,cm}_{1\,\%}$, 2500 $Lg^{-1}cm^{-1}$). For the different pepper varieties, the Lichtenthaler method showed carotenoid concentrations close to the HPLC results, as their predominant carotenoids (that is, capsanthin, 476 nm, $A^{1\,cm}_{1\,\%}$, 2200 $Lg^{-1}cm^{-1}$, data from supplier) are more similar to the mean absorption coefficients and wavelengths used by the Lichtenthaler method.

Compared to Lichtenthaler, it appears that the Hornero-Méndez method was more robust to wavelength deviations as the detection is based on 2 wavelengths, and a more complex carotenoid profile can be evaluated. Using this method also showed very accurate results for carrots and more similar results for the chlorophyll containing foods compared to HPLC. On the other hand, using the wavelength at 472 nm, the presence of lycopene led to an overestimation of the yellow fraction and a consequent overquantification of the total carotenoid content, resulting in 70% higher carotenoid estimates for tomatoes compared to HPLC.

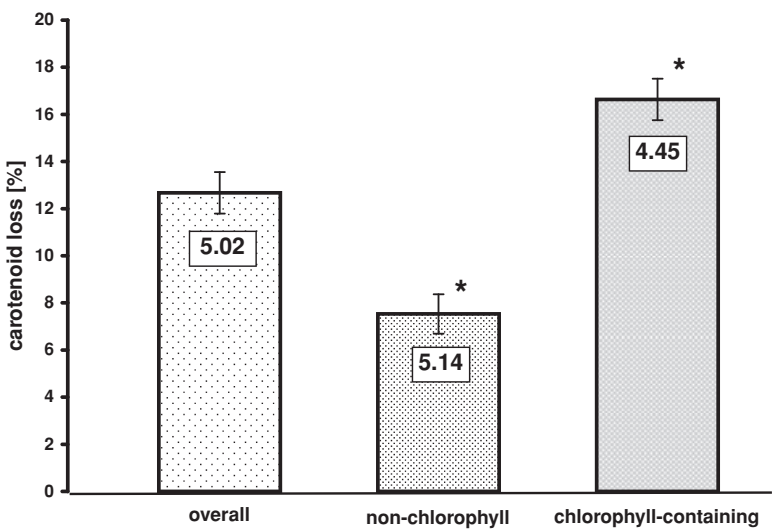

Figure 3—Carotenoid losses due to saponification (15% KOH, 15 min at RT) in different matrices. Chlorophyll containing foods showed greater susceptibility to carotenoid losses due to saponification than nonchlorophyll containing ones. The overall effect of saponification was 12.6 ± 0.9%. Values in chart represent mean carotenoid concentration of investigated species (mg/100 g). *Indicates statistical significant difference, $P < 0.001$ (Bonferroni).

Table 2— Carotenoid content of different fruits and vegetables ($n = 28$) according to 3 different spectrophotometric methods and HPLC. Values are given as mean ± SD (mg/100g edible portion).

	Carotenoid content ± SD (mg/100g edible portion)							
Food item	Lichtenthaler (unsaponified)		Method of Mean (saponified)		Hornero-Méndez (saponified)		HPLC (unsaponified)	
(A) Chlorophyll containing foods								
Apple* *Malus domestica*	0.59	0.05	0.51	0.07	0.46	0.23	0.21	0.01
Arugula *Eruca sativa*	11.30	0.07	9.53	0.28	10.85	0.37	10.36	1.89
Bean, green *Phaseolus vulgaris*	2.23	0.09	1.57	0.17	1.65	0.19	2.20	0.05
Blackberry* *Rubus fructiosus*	0.60	0.05	0.48	0.05	0.48	0.05	0.30	0.03
Broccoli *Brassica oleracea*	0.34	0.01	0.34	0.01	0.35	0.01	0.23	0.01
Endive *Cicorium endivia*	3.66	0.64	1.47	0.14	1.62	0.15	1.99	0.36
Grape* *Vitis vinifera*	0.09	0.01	0.10	0.02	0.08	0.05	0.11	0.02
Kale *Brassica oleracea*	3.04	0.04	1.95	0.27	2.07	0.03	2.00	0.29
Lamb's lettuce *Valerianella locusta*	12.33	0.73	10.31	0.64	11.63	0.72	7.70	0.60
Leek *Allium ampeloprasum*	1.79	0.06	1.29	0.07	1.40	0.08	2.62	0.45
Lettuce *Lactua sativa*	3.07	0.46	2.26	0.53	2.52	0.65	1.62	0.17
Pea *Pisum sativum*	3.44	0.30	2.88	1.16	3.13	1.33	3.79	0.12
Pepper, green *Capsicum annuum*	3.76	0.26	2.14	0.27	2.35	0.35	2.17	0.06
Spinach *Spinacia oleracea*	22.32	1.62	19.88	0.47	21.71	0.49	12.69	1.37
(B) Nonchlorophyll containing foods								
Apricot* *Prunus armeniaca*	5.85	0.29	5.31	0.65	5.55	0.84	4.39	0.6
Banana* *Musa* spec. *(Cavendish)*	0.11	0.01	0.11	0.01	0.11	0.01	0.35	0.11
Carrot *Daucus carota*	16.94	2.64	18.32	2.64	18.00	2.58	17.97	2.06
Cherries* *Prunus avium*	0.59	0.08	0.37	0.07	0.58	0.03	0.33	0.04
Corn *Zea mays*	0.81	0.10	1.13	0.12	0.83	0.09	1.06	0.15
Mandarin* *Citrus reticulata*	2.27	0.12	3.02	0.17	2.24	0.12	2.74	0.25
Melon, cantaloupe* *Cucumis melo*	3.01	0.23	2.98	0.52	2.15	0.37	3.25	0.59
Orange* *Citrus sinensis*	2.23	0.17	2.36	0.21	1.90	0.17	1.56	0.11
Peach* *Prunus persica*	1.30	0.03	1.36	0.05	1.23	0.04	1.32	0.05
Pepper, orange *Capsicum annuum*	8.05	0.29	6.79	0.91	7.24	1.17	9.57	0.79
Pepper, red *Capsicum annuum*	16.15	0.80	11.62	2.07	12.73	2.27	15.91	1.91
Pepper, yellow *Capsicum annuum*	4.79	0.63	4.02	1.43	4.82	1.74	6.90	1.68
Tomato *Solanum lycopersicum*	6.97	0.28	3.80	0.30	5.86	0.39	3.44	0.16
Watermelon* *Citrullus lanatus*	4.94	0.74	3.34	0.08	4.56	0.12	1.22	0.05
Mean ± SD	5.09^{A}	0.39	4.26^{B}	0.48	4.58^{B}	0.52	4.21^{B}	0.50
RSD (%)	7.58		11.19		11.44		11.83	
Chlorophyll-containing ± SD	5.23^{A}	0.32	$4.17^{B,C}$	0.30	4.60^{B}	0.34	3.68^{C}	0.39
Nonchlorophyll-containing ± SD	5.29^{A}	0.46	4.61^{B}	0.66	4.84^{B}	0.71	4.99^{B}	0.61^{B}
Fruits ± SD	1.96^{A}	0.16	0.81^{B}	0.17	0.74^{B}	0.19	0.61^{B}	1.42
Vegetables ± SD	7.12^{A}	0.54	5.84^{B}	1.81	6.40^{B}	1.76	6.01^{B}	0.17

*Considered as fruits; all other food items were considered as vegetables.
Different letters indicate significant differences ($P < 0.001$ for the difference between A, B, and C and $P < 0.01$ for the difference between B and C, Bonferroni).

The here presented Method of Mean was based on the assumption of the simultaneous presence of predominantly occurring carotenoids in fruits and vegetables (Khachik and others 1997; O'Neill and others 2001). The method exploits the mean absorption of these carotenoids at 450 nm, yielding inaccurate results if the majority of carotenoid species absorbed at more bathochrome (that is, capsanthin) or hypsochrome (that is, neoxanthin) wavelengths. Consequently, the Method of Mean underestimated red bell peppers by 27% compared to HPLC. The overestimation of lycopene was minimized by including the minor absorbance peak and absorption coefficient at 448 nm into eq. 2, which is close to the absorbance maxima of the other 4 carotenoids. However, when evaluated by mean carotenoid content and correlation, the Method of Mean was, on average, most close to results obtained by HPLC.

Taken together, the Lichtenthaler method yielded accurate results especially for fruits and vegetables of low chlorophyll content, but tended to overestimate matrices rich in chlorophyll and high in lycopene. In comparison, the method of Hornero-Méndez and Mínguez-Mosquera showed generally lower, more accurate results, with a good prediction for the majority of plant foods, with high lycopene concentrations still resulting in overestimation. The Method of Mean yielded similar results compared to the latter method with less overestimation of lycopene rich species, but underestimated carotenoid content in red and yellow pepper varieties due to a less common carotenoid profile.

Conclusions

Although originally not foreseen as a general quantification tool, the method according to Hornero-Méndez and Mínguez-Mosquera (including extraction modifications) could be used as a screening method for a broader variety of plant food items. Our in-house method, based on the mean absorption wavelength and coefficient of the 5 most abundant carotenoids in plants, can be used equally well, requiring less calculation steps, but has limitations if the food contains an unbalanced carotenoid profile, such as red bell peppers and watermelon. It appears that the typical overestimation by spectrophotometric methods due to minor compounds and degradation products is somewhat balanced by carotenoid losses due to saponification, resulting in a close estimation of the carotenoid content compared to HPLC.

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