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Resonance Raman Quantification of Nutritionally Important Carotenoids in Fruits, Vegetables, and Their Juices in Comparison to High-Pressure Liquid Chromatography Analysis

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A rapid nondestructive estimation of carotenoid levels in intact fruits and vegetables and their juices could have great value when selecting nutritionally valuable crops for further propagation and commercial use. Carotenoid levels of a variety of agricultural products and juices were measured using resonance Raman spectroscopy and compared to levels determined by extraction and high-pressure liquid chromatography. A strong correlation was observed between the two methods when evaluating juices and when comparing different strains of intact tomatoes at the same stage of ripening.

KEYWORDS: **β-Carotene; lycopene; lutein; zeaxanthin; Raman spectroscopy; HPLC**

INTRODUCTION

Many dietary fruits and vegetables are excellent sources of carotenoids such as β-carotene, lycopene, zeaxanthin, and lutein (*1*), nutrients that may play critical roles in the prevention of important human diseases such as cancer, cardiovascular disease (*2*−*4*), and age-related macular degeneration (*5*). Recent epidemiological and animal studies suggest a direct relationship between a high dietary intake of carotenoids from fruits and vegetables and a decreased risk of these disorders (*6*, *7*). To enhance agricultural and commercial production of products rich in carotenoids and to guide consumer choices of "healthy" fruits, vegetables, and juices, it is essential to develop rapid and reliable techniques to measure carotenoid levels. Currently, the most commonly used method to perform this screening is to bring the fruits and vegetables back to the laboratory for extraction and high-pressure liquid chromatography (HPLC) analysis (*8*−*10*), a very cumbersome, time-consuming, and expensive process.

Among various rapid detection technologies reported for carotenoid detection from intact fruits, resonance Raman spectroscopy holds promise. Raman detection allows one to use the strong and broad absorption bands of carotenoid molecules for resonant excitation in the fluorescence-free, longer wavelength spectral range at 488 nm for sensitive detection of the molecule's highly specific Raman response. The response is characterized by three strong, high-frequency, Stokes-shifted signals originating from carbon−carbon double-bond and single-bond stretches of the molecule's polyene backbone and from methyl bends with associated Raman peak positions at 1525, 1159, and 1008 cm^{-1}, respectively (*11*).

We have used resonance Raman spectroscopy as a sensitive and specific method for the noninvasive quantitative measurement of carotenoids in a variety of living human tissues such as the retina, skin, and oral mucosa (*12*−*14*). Although much more work needs to be done, these studies indicate that carotenoid resonance Raman spectroscopy might be a useful technique for the early detection of individuals who may be at risk for age-related macular degeneration or cancer later in life (*13*, *15*, *16*). We have also used our Raman instrumentation for the quantitative assessment of carotenoids produced in bacterial culture (*17*). Likewise, other researchers have reported that resonance Raman spectroscopy can be used to record carotenoid spectra from intact plant samples, but these studies have generally been qualitative in nature (*18*−*21*).

Carotenoid molecules are strong Raman scatterers; thus, nondestructive resonance Raman spectroscopy could be an extremely valuable biotechnological method for the rapid quantitative assessment of carotenoids. We report here a rapid screening method of carotenoid quantitation based on resonance Raman spectroscopy that could be used on intact agricultural products in the field or to continuously monitor juices during processing, and we compare the values obtained with carotenoid levels assessed by HPLC. A strong correlation was observed between the two methods when evaluating juices and when comparing different strains of intact tomatoes at the same stage of ripening.

MATERIALS AND METHODS

Selection of Fruits and Vegetables. A variety of fruits, vegetables, and juices were selected from local gardens and markets to represent

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Table 1. Fruits and Vegetables Selected for the Studies

fruits and vegetables (includes juices)	main carotenoids	major potential utility	ref
tomato (*Lycopersicon esculentum*)	lycopene, β-carotene, lutein	anti-prostate cancer	*3*
carrot (*Daucus carota)*	β-carotene, α-carotene	against many types of cancer, provitamin A supplement	*2*
orange (*Citrus sinensis)*	β-carotene,β-cryptoxanthin, lutein, α-carotene	anti-colon cancer	*24*
spinach (*Spinacia oleracea*)	lutein, zeaxanthin, β-carotene	against age-related macular degeneration	*5*
black grapes (*Vitis vinifera*)	β-carotene, lutein, α-carotene	provitamin A supplement, antioxidants	*2*

a diverse range of qualitative and quantitative levels of different carotenoids. These are listed in **Table 1**.

Standard Solutions. Stock solutions of carotenoid standards used in the study [β-carotene and lycopene (Sigma, St. Louis, MO), zeaxanthin (Hoffmann LaRoche, Basel, Switzerland), and lutein (Kemin Foods, Des Moines, IA)] were prepared in hexane. Concentrations of these standard solutions were checked spectrophotometrically using the corresponding extinction coefficients reported in a standard text (*22*). Aliquots were evaporated to dryness under an argon stream, and the residues were dissolved in the HPLC mobile phase and subjected to HPLC analysis. Standard calibration graphs were prepared for carotenoids by plotting peak area measurements at 450 nm vs concentration. Linearity, reproducibility, and recovery were determined periodically and found to be statistically significant ($R^2 = 0.95-0.98$, $P = 0.033-0.042$) for all of the major carotenoids analyzed.

Sample Preparation. All extraction work was done in the dark or in subdued light using a modified protocol based on a previously published method (*23*). Approximately 100 mg of fruit or vegetable tissue from at least three different samples was homogenized by grinding and then extracted in the presence of 2 mL of cold, oxygen-free acetone containing 0.1% (w/v) butylated hydroxytoluene to avoid promotion of epoxides. Acetone was chosen as the preferred solvent because it is miscible with water, it dissolves most carotenoids well, and it denatures any carotenoid−protein or carotenoid−lipid complexes. The samples were extracted until they became colorless. The acetone extracts were pooled and centrifuged to remove insoluble material. Whenever required (especially in the case of green vegetables), extracts were treated with 5 mL of 40% (w/v) methanolic potassium hydroxide solution and kept at 4 °C for 2 h for complete saponification of xanthophyll esters.

The combined acetone extract was transferred to a 50 mL centrifuge tube, and 5 mL of *n*-hexane (cold and oxygen-free) was added to it and mixed gently to avoid emulsion formation. Five milliliters of cold distilled water was then added to the mixture to cause phase separation. If necessary, a few drops of saturated NaCl solution were added to break an emulsion. The pigments in the organic phase were concentrated by vacuum evaporation and stored at −20 °C until they were analyzed. The pigments were reconstituted in 5 mL of HPLC mobile phase for HPLC analysis. Intensely colored extracts with high carotenoid levels were diluted as necessary to avoid overloading the HPLC system.

The pigment extraction from the fruit and vegetable juices was achieved using 1 mL aliquots of the fruit juice as a starting material. Prior to extraction, the pH of the acidic juices was adjusted to ∼7.0 using a concentrated sodium hydroxide solution. Five milliliters of methanol was then added, and the solution was centrifuged at 5000 rpm for 10 min. Saponification and organic extraction of the aqueous solution were performed as detailed above for plant tissues. The process was continued until a colorless aqueous layer was observed.

HPLC Analysis. The HPLC equipment (Thermo Separation Products Inc., San Jose, CA) had an autosampler, a two channel solvent degasser, a binary gradient pump, and a UV−visible photodiode array detector. HPLC grade solvents from Fisher Scientific (Pittsburgh, PA) were used as the mobile phases. The column was maintained at room temperature.

The chromatographic conditions for estimation of β-carotene and lycopene contents were similar to those reported earlier (*9*). The mobile phase was an isocratic mixture of acetonitrile:2-propanol:ethyl acetate (50:40:10 v/v) at a flow rate of 0.7 mL per minute. The analysis was performed on a reversed phase Luna C18(2) analytical column [250 mm length × 4.6 mm i.d. (Phenomenex, Torrance, CA); particle size, 5 μm; pore size, 100 Å].

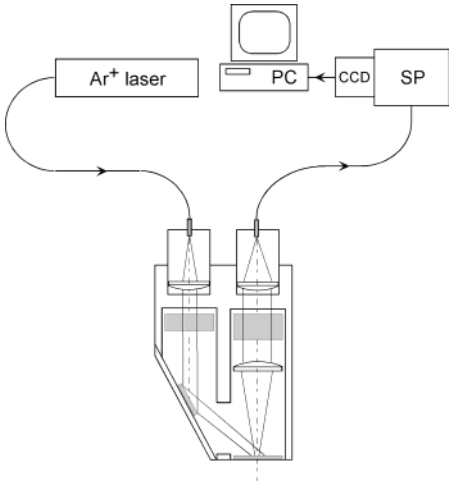

Figure 1. Schematics of the laser Raman detector for measurement of carotenoids in fruits and vegetables (not to scale). Laser excitation is routed via an optical fiber to the sample of interest. The Raman backscattered light is collimated by the probe module and sent via a different fiber to a spectrograph (SP) for light dispersion and detection with a CCD detector. A computer (PC) is used for instrument interfacing, data collection, and processing.

Lutein and zeaxanthin were separated using a cyano-HPLC column (*17*). Samples were dissolved in HPLC mobile phase [hexane: dichloromethane:methanol:N,N′-di-isopropylethylamine (80:19.2:0.7: 0.1 v/v)]. HPLC separation was carried out at a flow rate of 1.0 mL per min on a cyano column (Microsorb 250 mm length × 4.6 mm i.d., Varian Inc., Palo Alto, CA).

The columns were maintained at room temperature, and the HPLC detector was operated at 450 nm. The peak identities were confirmed by photodiode array spectra and by coelution with authentic standards as necessary. Integrated areas of the carotenoid peaks were converted to carotenoid concentrations through the use of calibration curves generated from HPLC injections of known quantities of authentic carotenoid standards. The carotenoid content estimated by HPLC correlated well with the values reported earlier in the literature (*10*).

Raman Measurements. Raman measurements did not require any preparation procedures with the exception of bringing the samples into contact with the window of the optical probe module of the system (**Figure 1**). The probe module and other components were identical to the system that we used previously for human skin measurements (*13*, *14*). For a typical measurement, a 5 mW argon laser light (488 nm) was directed as a 2 mm diameter spot onto the fruit or vegetable surface for 10 s.

Raman backscattered light was collected with a fiberoptic collection bundle and analyzed by a Raman spectrograph. The peak height at the carotenoid C═C stretch frequency of 1525 cm^{-1} was quantified after subtraction of the background fluorescence, using Windows-based software for data collection and processing (*13*). The Raman response was expressed as photon counts.

RESULTS AND DISCUSSION

Raman spectra of standard carotenoid solutions and selected plant tissues are shown in **Figure 2**. The three characteristic carotenoid Raman peaks originate from rocking motions of the

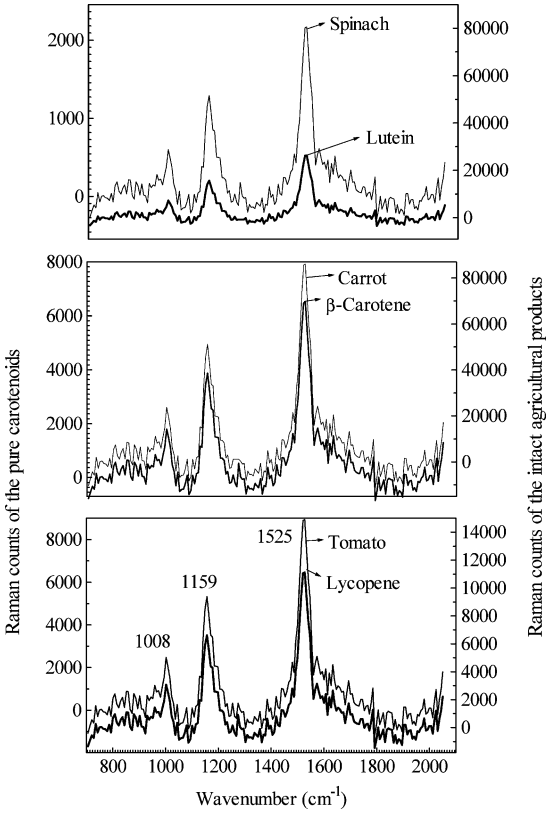

Figure 2. Resonance Raman spectra of carotenoids dissolved in tetrahydrofuran (lutein, β-carotene, and lycopene, left axis) as compared to agricultural products high in the respective carotenoids (spinach, carrot, and tomato, right axis).

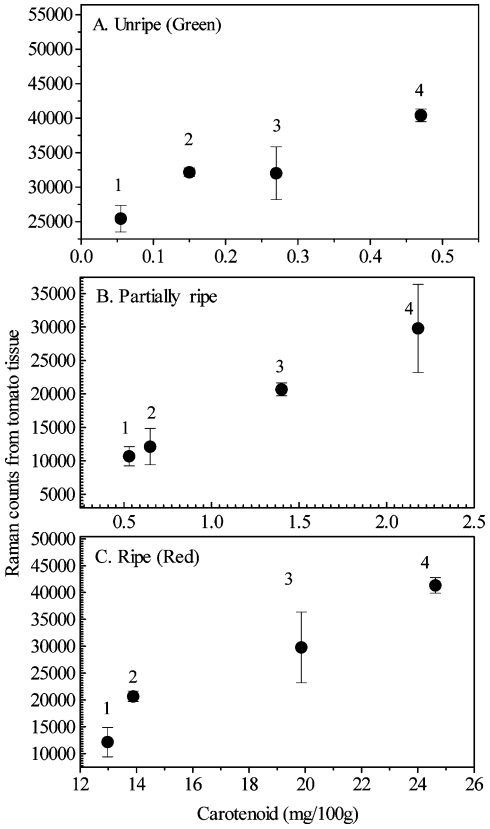

Figure 3. Comparison of HPLC-estimated carotenoid levels with the Raman responses obtained directly from the tomato skin (●) of different varieties of tomatoes at different ripening stages (1, cherry; 2, small salad; 3, large salad; and 4, Roma). All values are means ± SD for $n = 3$.

molecules' methyl components (1008 cm^{-1}), from carbon−carbon single-bond stretch vibrations (1159 cm^{-1}), and from carbon−carbon double-bond stretch vibrations (1525 cm^{-1}) of the polyene backbone (*11*). In all cases, the resonance Raman spectra from intact plant tissues precisely match the carotenoid standards regarding spectral locations and amplitudes of the peaks.

Chlorophylls are known to have moderate absorbance between 430 and 450 nm and to have strong absorption above 600 nm, and prior studies done with several types of chlorophyll report Raman peaks at and above 1606 (chlorophyll a) and 1650 cm^{-1} (chlorophyll d) (*11*, *12*). In this study, the carotenoid resonance Raman signal was detected at 527 nm, while excitation was performed at 488 nm, wavelengths where chlorophyll has practically no absorption and no chlorophyll Raman signals were detectable.

For comparison of HPLC and resonance Raman quantitation of carotenoids, we chose to focus our attention primarily on carrots and tomatoes because they have relatively simple carotenoid profiles, in contrast to green leafy vegetables. The results are listed in **Table 2**. Within the two categories of agricultural product, there is a definite trend of increasing Raman response with greater carotenoid content as measured by HPLC. Spinach leaves, however, had Raman counts that were much higher than carrots or tomatoes (~125 000 Raman counts), despite having substantially lower total carotenoid contents on a per weight basis. This suggests that the very different optical absorption characteristics of tomatoes, carrots, and leafy vegetables preclude a direct comparison of carotenoid levels by Raman spectroscopy, although it appears reasonable to perform such quantitative comparisons on samples within a particular category of agricultural product. To examine this phenomenon further, we measured four different types of tomato (cherry, small salad, large salad, and Roma) at various stages of ripening. The results are displayed in **Figure 3** and indicate that the

Table 2. Carotenoid Content of Tomatoes and Carrots as Measured by HPLC and by Resonance Raman Spectroscopy[a]

	carotenoid content by HPLC (μg/100 g)				total carotenoids (μg/100 g)	Raman intensity at 1525 cm^{-1} (counts)
	lutein	zeaxanthin	β-carotene	lycopene		
			red tomato			
cherry	190 ± 8	27 ± 4	2016 ± 100	9767 ± 12	12 000	12 507 ± 123
small salad	190 ± 5	3 ± 1	5784 ± 32	7923 ± 65	13 900	20 806 ± 45
large salad	700 ± 3	50 ± 5	6953 ± 43	12 165 ± 72	19 868	29 856 ± 123
Roma	480 ± 12	13 ± 7	5674 ± 21	18 499 ± 96	24 666	41 482 ± 146
			carrot			
light orange	260 ± 11		17 000 ± 47		17 260	23 387 ± 234
dark orange	450 ± 12		21 000 ± 43		21 450	60 164 ± 123

[a] All values are means ± SD for $n = 3$.

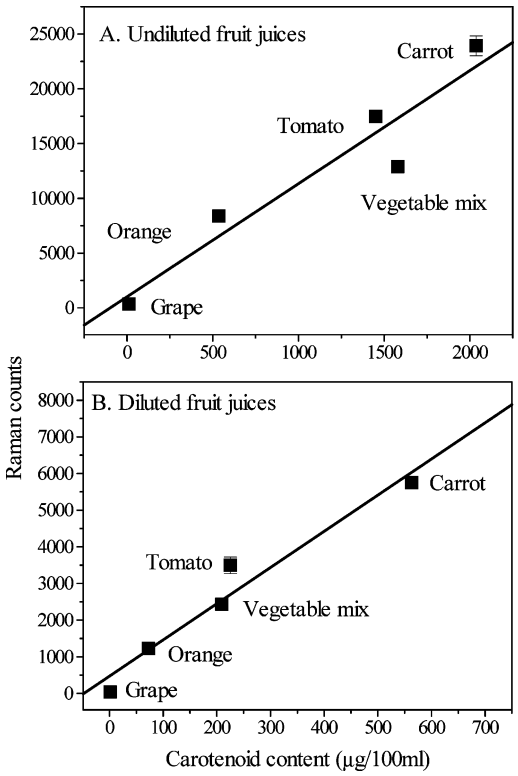

Figure 4. Correlation of HPLC-estimated carotenoid levels with the Raman responses measured from (**A**) undiluted and (**B**) diluted (1:10) fruit juices. All values are means ± SD for $n = 3$.

Raman signals recorded from the surface of the tomatoes at the same stage of ripeness were directly proportional to HPLC measurements of carotenoid levels in fully ripe red tomatoes ($R^2 = 0.96$, $P = 0.014$), partially ripe tomatoes ($R^2 = 0.986$, $P = 0.0002$), and unripe green tomatoes ($R^2 = 0.90$, $P = 0.045$), but we noted unusually high Raman signals for the unripe tomatoes relative to the fully ripe tomatoes. We suspect that poor laser penetration and self-absorption of the Raman scattered light by the extraordinarily high concentrations of lycopene and β-carotene in intact ripe tomatoes may explain the low Raman responses relative to green tomatoes

Fruit juices were selected for further studies because their high water content lowers the concentration of absorbing chromphores, thus lessening the problem of self-absorption, and they can be measured under standardized optical conditions using 1 mm path length quartz cuvettes. Fruit juices with varying levels and types of carotenoids were used to determine the utility of Raman spectroscopy for their quantitation. HPLC analysis revealed that carrot, tomato, and orange juices were rich in β-carotene, lycopene, and β-cryptoxanthin, respectively, while grape juice had very low carotenoid levels and a vegetable juice mix was intermediate. A direct correlation between the total carotenoid content (as estimated by HPLC) and the Raman signals was seen at a wide range of carotenoid levels naturally found in these juices (**Figure 4A**), but some undiluted samples were high in particulate matter, making the samples turbid and highly scattering. To improve the quality of the juice Raman measurements, we therefore diluted the samples with water to lower both scattering and optical density (**Figure 4B**). The total carotenoid content measured by HPLC and Raman responses, respectively, was well-correlated and statistically significant for diluted ($R = 0.98$, $P = 0.003$) and undiluted juices ($R = 0.94$, $P = 0.01$).

Our initial survey of the potential value of resonance Raman spectroscopy for the assessment of carotenoid levels in fruits, vegetables, and juices demonstrates great potential in some areas and limitations in others. The test is rapid and nondestructive, and the instrument, although custom-assembled, utilizes components that are less expensive in aggregate than a gradient HPLC system with photodiode array detection. The instrument could certainly be modified for portable use in the field or for flow-through testing in a juice production operation. The test, however, is generally unable to separate and distinguish between the various carotenoids that may be present. Laser penetration is hampered by optically dense media, so the depth of penetration of the laser light will be different for each class of agricultural product, and the Raman response appears to vary dramatically at different stages of ripening. On the other hand, there does seem to be a good correlation between our Raman measurements and the total carotenoid content measured by HPLC when comparing members of the same class of agricultural product (i.e., different varieties of tomato) at identical stages of ripening, and measurements worked particularly well on juices, although dilution and/or clarification may be required for optimal Raman readings. Thus, although it is clear that resonance Raman spectroscopic measurements of carotenoid levels in nutritionally important fruits and vegetables will never replace HPLC as the "gold standard", our instrument could serve as an easily used device for monitoring carotenoid-enhanced juice production or for field selection of carotenoid-enriched agricultural products.

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