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Isotopic Labeling and LC-APCI-MS Quantification for Investigating Absorption of Carotenoids and Phylloquinone from Kale (*Brassica oleracea*)

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The ability to study bioavailability of nutrients from foods is an important step in determining the health impact of those nutrients. This work describes a method for studying the bioavailability of nutrients from kale (*Brassica oleracea* var. Acephala) by labeling the nutrients with carbon-13, feeding the kale to an adult volunteer, and analyzing plasma samples for labeled nutrients. Results showed that conditions for producing atmospheric intrinsically labeled kale had no detrimental effect on plant growth. Lutein, β-carotene, retinol, and phylloquinone were analyzed using liquid chromatography−atmospheric pressure chemical ionization mass spectrometry. Analysis of plasma samples showed that labeled lutein peaked in plasma at 11 h (0.23 μM), β-carotene peaked at 8 (0.058 μM) and 24 h (0.062 μM), retinol peaked at 24 h (0.10 μM), and phylloquinone peaked at 7 h (3.0 nM). This method of labeling kale with ^{13}C was successful for producing clearly defined kinetic curves for ^{13}C-lutein,^{13}C-β-carotene, ^{13}C-retinol, and ^{13}C-phylloquinone.

KEYWORDS: Carotenoid; *β*-carotene; lutein; retinol; vitamin A; phylloquinone; vitamin K; isotope; label; mass spectrometry; LC-MS; kale; *Brassica oleracea*

INTRODUCTION

Phytonutrients not only help individuals to meet daily requirements but also can improve health by protecting against chronic diseases, including cancer, cardiovascular disease, and age-related macular degeneration (*1*−*5*). Carotenoids are a large class of compounds that may contribute to the health-promoting effects of plant-based foods by acting as antioxidants in the defense against oxidative stress and free radicals (*6*−*8*). Some carotenoids, such as β-carotene, are also precursors to vitamin A, therefore making plant-based foods a possible contributor to vitamin A status in humans. Phylloquinone (vitamin K_1) is required for carboxylation of factors essential for blood-clotting (*1*).

Regardless of the nutrient content of a plant-based food, the ability of those plant-derived nutrients to impart health benefits depends on the gastrointestinal tract's ability to extract the nutrients from the plant material. The presence of a specific nutrient in a plant-derived food is insufficient for providing health benefits if the bioavailability of that nutrient is very low. For example, spinach contains a high calcium content, but the presence of phytates and oxalates in spinach reduces the efficiency of calcium absorption from the gastrointestinal tract (*9*−*11*). Thus, to understand a nutrient's ability to promote health, it is critical to clearly understand the nutrient's bioavailability from food sources.

Methods for studying bioavailability generally fall into two categories: measuring the portion of an ingested nutrient that crosses the intestinal barrier by sampling plasma and/or biological tissues or measuring the portion of an ingested nutrient that appears in the feces (then comparing the mass of nutrient in feces to the total dose given). A difficulty arises in the plasma sampling method when newly ingested nutrient mixes with endogenous circulating nutrient. In that case, a label or tag is needed to identify the newly absorbed nutrient. A difficulty also arises in the fecal sampling ("balance") method because it is possible for nutrients to pass through the small intestine unabsorbed and become metabolized by the intestinal flora, thus leading to overestimation of nutrient bioavailability, and it is also possible for the intestinal bacteria to synthesize some nutrients in the colon (such as biotin, pantothenic acid, and vitamin K), leading to underestimation of nutrient bioavailability. Stable isotope methodology solves some of the problems encountered by the other methods because administering an isotope-labeled nutrient to an individual allows discernment of that nutrient from the endogenous nutrient in biological samples.

Stable isotopes have been used to study bioavailability of carotenoids (*12*−*22*) and phylloquinone (*23*) previously. Studying the bioavailability of nutrients labeled with stable isotopes requires sophisticated chromatography and mass spectrometry. Recent advances in analytical chemistry have led to the development of GC-MS and/or LC-MS methods for quantifying

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plasma levels of isotopically labeled carotenoids (*17*, *24*−*29*), retinol (*27*−*31*), and phylloquinone (*23*, *32*).

This paper describes a method of studying the bioavailability of nutrients from kale by labeling the nutrients in the plant tissue with carbon-13, administering the labeled vegetables to an adult volunteer, and analyzing plasma samples for lutein, β-carotene, retinol, and phylloquinone using LC-APCI-MS.

MATERIALS AND METHODS

Intrinsic Labeling of Kale. Kale (*Brassica oleracea* var. Acephala cv. Vates) was labeled uniformly with ^{13}C by growing plants continuously in an atmosphere containing carbon-13 dioxide ($^{13}CO_2$) starting approximately 8 days after sowing. At the start of labeling, the cotyledons (seed leaves) had expanded and the primary leaves (first true leaves) were beginning to unfold in the majority of plants. A relatively uniform population was selected at this stage by thinning plants with poor growth or late germination.

The apparatus for labeling consisted of a clear acrylic box (Plexiglas G, Rohm and Haas, Wilmington, DE) with a separate base (6.35 mm thick) and upper enclosure (3.18 mm thick with interior dimensions (L × W × H) of 1.12 m × 0.99 m × 0.61 m and total volume of 676 L). The upper enclosure fit into a water-filled trough in the base to form an airtight seal. The acrylic box was located inside a larger controlled environment chamber. The air temperature inside the enclosure was maintained passively at 24−25 °C by regulating the exterior temperature in the environmental chamber at 17 °C. A small muffin fan (model 4C550, Dayton, Niles, IL) was mounted centrally 0.04 m below the upper surface of the enclosure to circulate air upward and over the inside surface for mixing and heat exchange. A coldfinger inside the enclosure (connected to a source of 10 °C water) controlled humidity at approximately 90% and eliminated condensation on the walls of the enclosure. Condensate from the coldfinger drained into the water trough. Temperature and humidity inside the labeling chamber were monitored with a digital meter (Solomat, Norwalk, CT).

The environmental chamber provided continuous light for photosynthesis from a 50:50 mix of 400 W metal halide and high-pressure sodium lamps (Sylvania-GTE, Danvers, CT). The walls of the chamber were lined with reflective aluminum panels to improve light distribution. Photosynthetically active radiation (400−700 nm) was measured with a quantum sensor (LiCor, Lincoln, NE) and was determined to be 900 μmol m^{-2} s^{-1} incident on the acrylic enclosure and between 600 and 800 μmol m^{-2} s^{-1} inside the enclosure at plant canopy height depending on internal shade and plant stature. For comparison, instantaneous light levels in the enclosure were about one-third of midlatitude peak values at the summer solstice, while daily integrals were about 25% greater than maximum average values outdoors (*33*). The 24 h photoperiod accelerated plant growth and avoided the need to recover respired $^{13}CO_2$ that would otherwise have been lost during nightly dark periods.

Approximately 20 plants were raised in each of four bins (0.43 m × 0.52 m × 0.15 m, W × L × D; Nalgene) filled with vermiculite (ca. 30 L each). Plants were watered 1−2 times daily with a complete nutrient solution (*34*) applied either from above during germination or by subirrigation after the roots became established. The subirrigation system consisted of a manually operated submersible pump that filled the base of the enclosure for a predetermined depth and time and a manually operated drain. Water seals prevented outside air from mixing with the interior atmosphere during labeling.

Air within the labeling chamber was sampled (0.5 L/min) using separate ports on top of the chamber adjacent to the muffin fan and monitored for CO_2 concentration with an infrared gas analyzer (model WMA-3, PP Systems, Haverhill, MA). The analyzer operated in conjunction with a PID controller (model CN76000, Omega, Stamford, CT) and a solenoid value to inject $^{13}CO_2$ at greater than 99% g atom ^{13}C (Isotec Inc., Miamisburg, OH) through a separate port on top of the enclosure on the other side of the muffin fan. CO_2 levels in the enclosure were maintained at 400 ppm. Because the analyzer was much less sensitive to $^{13}CO_2$ than $^{12}CO_2$, it was calibrated using a known concentration of $^{13}CO_2$ in N_2 gas prepared using a volumetric gas mixing apparatus (Digamix G18, Woesthoff, Bochum, Germany). The tightness of the water seal was judged by monitoring CO_2 concentrations inside the box after adding or depleting CO_2. Levels were constant for at least 24 h, indicating no exchange between the interior and the exterior of the enclosure. Briefly opening a valve on the box, however, caused a rapid change in CO_2 concentration to a new steady state demonstrating rapid mixing and equilibration of atmospheric gas concentrations. CO_2 consumption was monitored during plant growth.

Labeling was terminated at canopy closure, which coincided with a decline in the rate of increase of CO_2 consumption. Plants were harvested rapidly in dim light after opening the enclosure and stored on ice in sealed plastic bags. Primary leaves (partially expanded at the start of labeling) and roots were not included in the material harvested for feeding trials. Material for the feeding study was cleaned, weighed, and blanched. The blanched kale was chopped into 1 in. × 1 in. pieces, mixed for batch uniformity, and frozen at −80° C until prepared for consumption.

Subject and Treatment. As proof of concept, a healthy 84 kg man consumed a single serving of ^{13}C-kale, and bioavailability of selected ^{13}C nutrients was assessed as plasma response. The subject gave written informed consent to participate in this study, which was approved by the Johns Hopkins University Bloomberg School of Hygiene and Public Health, Committee on Human Research. Four hundred grams of kale was warmed by microwave and consumed with 30 g of peanut oil. The kale dose provided the subject with 34 μmol of β-carotene (equivalent to 18 mg of unlabeled β-carotene), 33 μmol of lutein (equivalent to 19 mg of unlabeled lutein), and 156 nmol of phylloquinone (equivalent to 70 μg of unlabeled phylloquinone). Blood samples were collected through an indwelling catheter approximately hourly the first day, daily the first week, and biweekly for the next 5 weeks after the kale was consumed. Blood samples were centrifuged at 15 000*g* for 20 min at 4 °C. The plasma was aliquotted and frozen at −80 °C until prepared for analysis.

For 1 week prior to consumption of the kale treatment and during sample collection, the subject consumed a controlled diet. The subject was instructed to consume only and all foods provided by the Beltsville Human Nutrition Research Center. The diet contained 15% of energy from protein, 32% from fat, and the balance from carbohydrate. The diet provided 2 mg of β-carotene per day and 6 mg of total carotenoids per day. During the day of kale ingestion, the subject consumed foods free of both β-carotene and vitamin A.

Quantification of Labeled and Unlabeled Compounds in Plant Tissue and Biological Samples Reagents. Hexane, EtOH, chloroform, 2-propanol, MTBE, and MeOH were purchased from Fisher Scientific. Lutein was purchased from Extrasynthase (Genay, France). Phylloquinone, β-apo-8′-carotenal, β-carotene, retinol, and BHT were purchased from Sigma Chemical Co. (St. Louis, MO). β-Carotene-d8 (10,10′,19,19,19,19′,19′,19′-[$^2H_8$]-all *trans*-β-carotene) and retinyl acetate-d8 (10,19,19,19,14,20,20,20-[$^2H_8$]-retinyl acetate) were purchased from Cambridge Isotope Labs (Woburn, MA). $^{13}CO_2$ (>99% carbon-13) was purchased from Isotec, Inc.

Kale Extraction. The extraction procedure was carried out under subdued lighting. Kale was ground into a fine powder with liquid nitrogen using a mortar and pestle. Three grams of powder was weighed into a 25 mL test tube for extraction. The sample was extracted in duplicate. Nine milliliters of 0.1% BHT in EtOH was added to each sample, and samples were vortexed for 2 min to precipitate proteins. Then, 9 mL of hexane:toluene (5:4 v/v) was added, and samples were vortexed and placed in a 70 °C water bath for 10 min. After 10 min, samples were removed from the water bath, 180 μL of 80% KOH was added, and samples were vortexed and returned to the water bath for an additional 15 min. Samples were vortexed halfway through the 15 min saponification. Samples were removed from the water bath and placed on ice where 3 mL of ddH_2O was added before vortexing. Samples were then centrifuged at 1140*g* for 10 min, and the organic layer (top) was collected to a separate test tube. The hexane:toluene (10:8 v/v) extraction was repeated twice more with 6 mL of the organic mixture. A 100 μL fraction was taken from the combined organic layer (total 21 mL) and added to 50 μL of 0.5 μg/mL β-carotene-d8 and 0.356 μg/mL β-apo-8′-carotenal. The samples were then dried under N_2 and reconstituted in 200 μL of MTBE:MeOH (1:1 v/v) prior to injection onto the LC-MS. LC-MS conditions are described below.

Plasma Sample Preparation. Plasma extraction was performed under subdued lighting. Phylloquinone, $^{13}C_{31}$-phylloquinone, retinol, $^{13}C_{20}$-retinol, and all carotenoids were extracted from 0.5 mL of plasma. The plasma was thawed, and 50 μL of β-apo-8′-carotenal (0.356 μg/mL) and retinol-d8 (saponified from retinyl acetate-d8) (0.511 μg/mL) was added as internal standards before addition of 0.5 mL of EtOH. Samples were extracted twice with 1.5 mL of hexane. The combined hexane extracts were dried under N_2 and reconstituted in 200 μL of MTBE:MeOH (1:1 v/v).

Because of coelution of $^{13}C_{40}$-β-carotene (*m*/*z* 577) with an unknown metabolite (*m*/*z* 577) following the above extraction procedure, an additional plasma aliquot (0.5 mL) was extracted and subjected to SPE to separate the $^{13}C_{40}$-β-carotene from the interfering metabolite (*35*). The beginning of the extraction was the same as above, except that 50 μL of β-carotene-d8 (0.5 μg/mL) was added as internal standard. After they were dried, the samples were reconstituted in 200 μL of chloroform and applied to Strata NH_2 SPE cartridges (Phenomenex, Torrance, CA) preconditioned with 2 mL of hexane. The samples were pulled into the column and eluted with 3 mL of chloroform:2-propanol (2:1 v/v). Samples were dried under N_2, reconstituted in 200 μL of hexane, and applied to another preconditioned (2 mL of hexane) SPE cartridge. β-Carotene was eluted with 4 mL of hexane, dried under N_2, and reconstituted in 200 μL of MTBE:MeOH (1:1 v/v) prior to injection on the LC-MS.

LC-MS Conditions. The LC-MS consisted of an Agilent HP series 1100 G1946A MSD with an APCI source connected to a G1315A DAD, cooled column compartment, cooled autosampler, automatic solvent degasser, binary pump, YMC C_{30} column (3 μm, 250 mm × 4.6 mm), and C_{30} guard cartridge. The MSD source was positive APCI with the spray chamber gas temperature set at 350 °C, vaporizer temperature set at 400 °C, nitrogen nebulizer pressure set at 45 psig, and corona current set at 5 μA. At time 0.0 min, the VCap was set to 3800 V and the drying gas (nitrogen) was 7 L/min. At 7.5 min, the VCap changed to 3600 V and the drying gas changed to 6 L/min. SIM was used to record abundance of protonated molecules of unlabeled and $^{13}C_{40}$-lutein (*m*/*z* 551 and 591, respectively; after loss of water) and β-carotene (*m*/*z* 537 and 577), as well as unlabeled and $^{13}C_{31}$-phylloquinone (*m*/*z* 451 and 482), unlabeled retinol, retinol-d8, and $^{13}C_{20}$-retinol (*m*/*z* 269, 276, and 289), and unlabeled β-apo-8′-carotenal (*m*/*z* 417) and β-carotene-d8 (*m*/*z* 545). Data were collected using Agilent Chemstation software. Molar concentrations of labeled and unlabeled lutein and phylloquinone were calculated using β-apo-8′-carotenal as an internal standard; retinol-d8 was used to calculate labeled and unlabeled retinol, and concentrations of labeled and unlabeled β-carotene were calculated using β-carotene-d8 as an internal standard. The DAD was set to collect signals at 450 and 325 nm with bandwidths of 100 and scan 250−540 nm with a range step of 2 nm and threshold set at 1.00 mAU.

The solvent system was the same as that used by Wang et al. (*27*) and consisted of solvent A (1 mM ammonium acetate in MeOH) and solvent B (MTBE). The gradient was 15% solvent B at 0 min to 30% B at 12 min, which was held until 18 min, when solvent B was increased to 100% and held until 23.0 min at which time B was decreased back to 15% and the column was equilibrated for 10 min. The flow rate was 1 mL/min, and the injection volume was 50 μL. The method was validated for phylloquinone, β-carotene, lutein, and retinol against a NIST standard reference material (SRM 968C, Gaithersburg, MD).

RESULTS

Labeled kale was prepared in two separate runs of 7 and 9 days and yielded total shoot fresh weights (above the primary leaves) of 1541 (70 plants) and 1767 g (80 plants), respectively (approximately 22 g fresh weight per shoot). Labeling was initiated 8 days after sowing. Duration was determined by canopy closure. No abnormalities were observed in plant morphology after replacement of $^{12}CO_2$ with $^{13}CO_2$. For comparison, two runs lasting 9 and 10 days using $^{12}CO_2$ yielded 2449 and 2026 g (80 plants each), respectively. Individual shoot fresh weights were 30.6 and 25.3 g. Dry matter averaged 9.2% of fresh weight for ^{12}C plants (not determined for ^{13}C). Although fresh weights were greater for ^{12}C plants, note that growth duration was slightly longer. On the other hand, ^{13}C plants had relatively high rates of photosynthesis. Maximum rates of CO_2 assimilation (mmol $^{13}CO_2$ or $^{12}CO_2$ per day, per plant; determined gravimetrically) were 26 and 27 for ^{13}C plants and 23 and 18 for ^{12}C plants. β-Carotene content of the ^{13}C-kale was 4.9 mg/100 g, lutein content was 5.1 mg/100 g, and phylloquinone content was 18.8ug/100 g.

The mass spectrum, in positive ion APCI, showed a base peak for unlabeled lutein at *m*/*z* 551, corresponding to the protonated molecule after loss of water. Unlabeled β-carotene had a base peak at *m*/*z* 537. The base peak for unlabeled retinol was at *m*/*z* 269, also corresponding to the protonated molecule after loss of water. The base peak for unlabeled phylloquinone was the protonated molecule with a *m*/*z* of 451. A linear response for all compounds was found over the range from 7.8 ng/mL to 2 μg/mL, given a 50 μL injection, with $R^2 > 0.99$. In molar terms, this corresponds to the following linear ranges: for β-carotene, 730 fmol to 190 pmol; for lutein, 690 fmol to 175 pmol; for phylloquinone, 865 fmol to 220 pmol; for retinol, 1.35−350 pmol. The limit of detection at a signal-to-noise ratio of 3 was 90 fmol for lutein, 90 fmol for β-carotene, 170 fmol for retinol, and 3 fmol for phylloquinone. Labeled and unlabeled nutrients had nearly the same retention time in the MS, in accord with the findings of Wang et al. (*27*) for labeled ($^{13}C_{10}$) vs unlabeled β-carotene, indicating that the labeled and unlabeled nutrients have similar physical properties.

Analysis of the mass chromatogram of the extract of ^{13}C-labeled kale showed that the base peak for labeled lutein was at *m*/*z* 591 and the base peak was at *m*/*z* 577 for labeled β-carotene, which represent the completely labeled molecules ($^{13}C_{40}$). The base peak for labeled phylloquinone was at *m*/*z* 482, also representing the completely labeled molecule ($^{13}C_{31}$). Total lutein from labeled kale was comprised of 54% $^{13}C_{40}$, 30% $^{13}C_{39}$, 10% $^{13}C_{38}$, 3% $^{13}C_{37}$, and 0.6% unlabeled lutein. Total β-carotene was comprised of 52% $^{13}C_{40}$, 29% $^{13}C_{39}$, 11% $^{13}C_{38}$, 3% $^{13}C_{37}$, and 0.04% unlabeled β-carotene, and total phylloquinone was 55% $^{13}C_{31}$, 27% $^{13}C_{30}$, 9% $^{13}C_{29}$, 4% $^{13}C_{28}$, and 0.6% unlabeled phylloquinone. LC-MS analysis of duplicate kale extracts showed that the subject consumed 33 μmol of labeled lutein, 34 μmol of labeled β-carotene, and 156 nmol of labeled phylloquinone.

LC-MS-extracted ion chromatograms of labeled and unlabeled baseline and 8 or 24 h lutein, β-carotene, retinol, and phylloquinone are shown in **Figures 1−4**. As expected, baseline chromatograms for all nutrients showed no ions in the channel where one would expect the ^{13}C analytes.

Labeled lutein (**Figure 5**) derived from the kale dose was detectable in the subject's plasma for 28 days following the kale dose. Plasma ^{13}C-lutein peaked at 0.23 μM at 11 h following the kale dose. Average plasma unlabeled lutein level was 0.28 μM during the study period. Thus, the labeled lutein reached 82% of the plasma unlabeled lutein level at the peak concentration.

Labeled β-carotene (**Figure 6**) from the kale was detectable in the subject's plasma for the full 46 day sampling period. ^{13}C-β-Carotene showed two peaks: one at 8 h (0.058 μM) and one at 24 h (0.062 μM), gradually decreasing to 0.0036 μM by 1080 h (46 days). The average plasma unlabeled β-carotene level was 0.297 μM during the study period. Therefore, the labeled β-carotene reached 20 and 21% of average unlabeled β-carotene at 8 and 24 h, respectively.

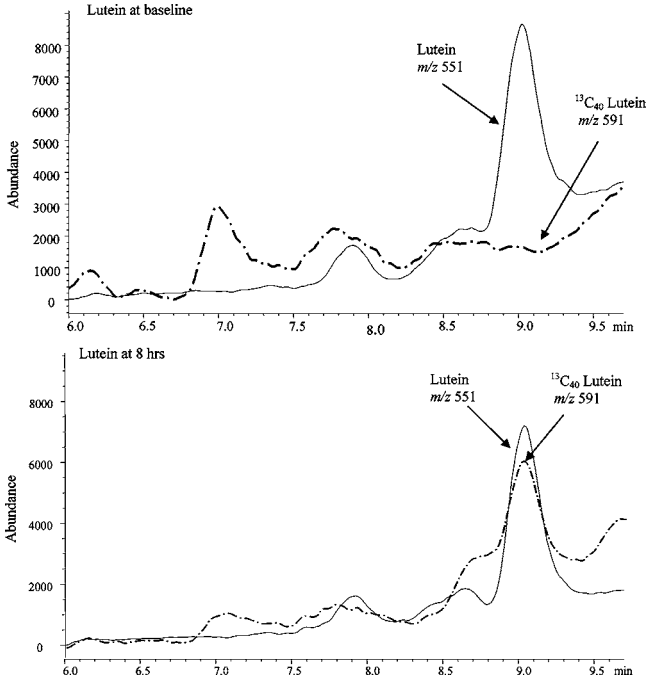

Figure 1. Extracted ion chromatogram tracings for lutein (*m/z* 551 representing $(M + H - H_2O)$) and $^{13}C_{40}$-lutein (*m/z* 591) from before kale consumption (baseline) and at the 8 h time point after a subject consumed 400 g of ^{13}C-kale.

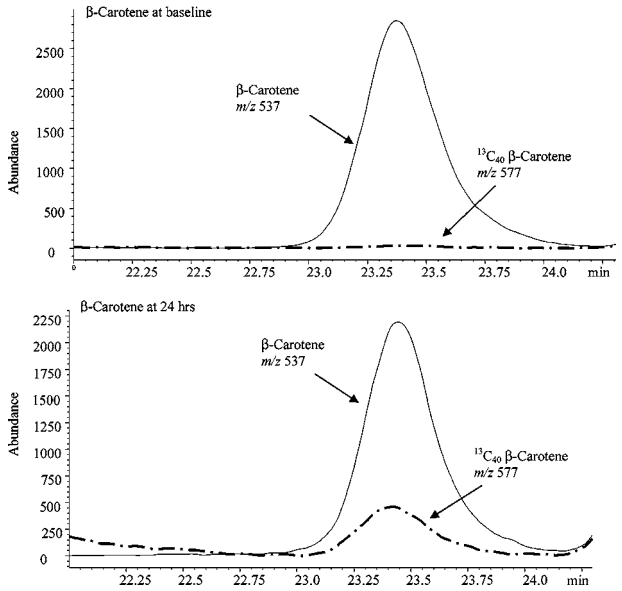

Figure 2. Extracted ion chromatogram tracings for β-carotene (*m/z* 537 representing $(M + H)^+$) and $^{13}C_{40}$-β-carotene (*m/z* 577) from before kale consumption (baseline) and at the 24 h time point after a subject consumed 400 g of ^{13}C-kale.

Labeled retinol can be assumed to have been derived from labeled β-carotene in the kale dose, since β-carotene was the only provitamin A carotenoid present in the kale. ^{13}C-Retinol was also detected throughout the entire 46 day sampling period. Labeled retinol peaked at 24 h (0.10 μM) and then gradually decreased to 0.023 μM by day 46. The average plasma unlabeled retinol level was 3.0 μM during the study period. Therefore, the labeled retinol reached 3% of unlabeled retinol at the peak concentration.

Labeled phylloquinone was detectable for only 7 days following the kale dose. Similar to β-carotene, the phylloquinone peaked at 7 h (3.0 nM), but in contrast to β-carotene,

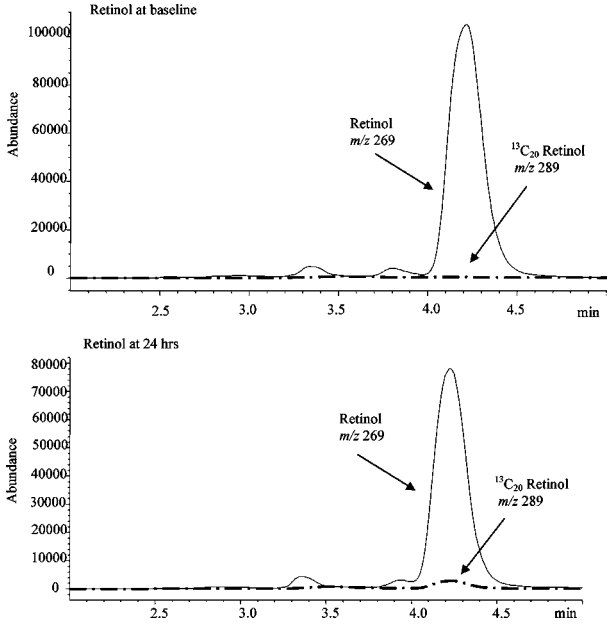

Figure 3. Extracted ion chromatogram tracings for retinol (*m/z* 269 representing $(M + H - H_2O)$) and $^{13}C_{20}$-retinol (*m/z* 289) from before kale consumption (baseline) and at the 24 h time point after a subject consumed 400 g of ^{13}C-kale.

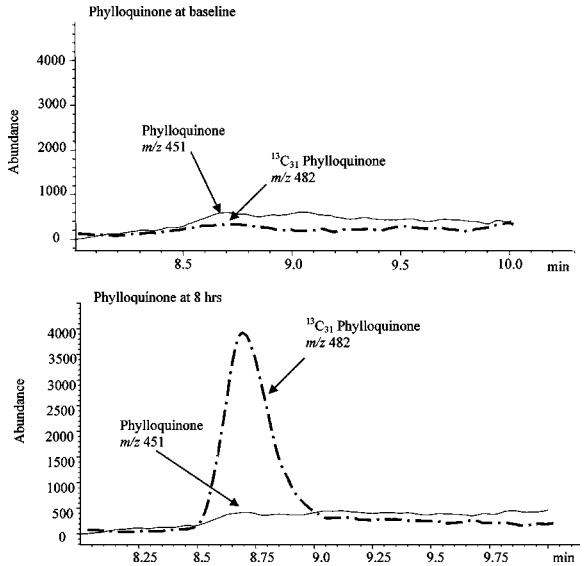

Figure 4. Extracted ion chromatogram tracings for phylloquinone (*m/z* 451 representing $(M + H)^+$) and $^{13}C_{31}$-phylloquinone (*m/z* 482) from before kale consumption (baseline) and at the 8 h time point after a subject consumed 400 g of ^{13}C-kale.

phylloquinone showed only a single peak. At the peak, ^{13}C-phylloquinone was 12-fold greater than average unlabeled phylloquinone.

DISCUSSION

As most of the nutrients that humans consume come from a variety of food sources and not supplements, it is important to understand how bioavailable those nutrients are from different food sources. Intrinsic labeling of nutrients in plants is a key technique for studying bioavailability so that recently ingested nutrients may be discerned from endogenous nutrients in biological samples. A variety of techniques have been used for labeling minerals in plants, including enrichment of nutrient solution (*36*−*40*), stem injection (*40*−*42*), and foliar application

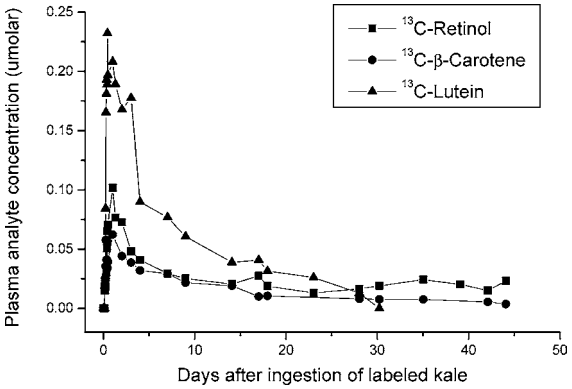

Figure 5. Plasma ^{13}C-lutein (μM), ^{13}C-β-carotene (μM), and ^{13}C-retinol (μM) as a function of time after ingestion of 400 g of ^{13}C-kale.

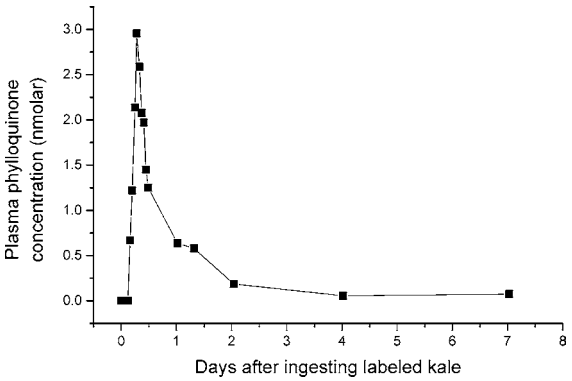

Figure 6. Plasma ^{13}C-phylloquinone (nM) as a function of time after ingestion of 400 g of ^{13}C-kale.

(*40*, *42*). For labeling of organics in plants for bioavailability studies, deuterium oxide enrichment of the nutrient solution has been investigated by Grusak (*23*, *43*). The deuterium is taken up by the roots and incorporated into molecules throughout the plant. The drawback of this method is that high levels of deuterium enrichment can be deleterious to plant growth. Deuterium enrichment above 50 atom percent excess results in stunted growth rates (*43*). For this reason, only partial random labeling can be achieved with deuterium, complicating laboratory analysis.

The additional alternative for intrinsically labeling nutrients in plants is to present the label to the plant atmospherically. Exposing a plant to labeled carbon dioxide allows organic molecules to be labeled as the carbon is fixed photosynthetically and distributed to tissues through biosynthetic pathways. The specialized equipment required, including a gastight chamber and special instruments for gas handling and analysis, makes this method fairly difficult. Furthermore, uniformly labeling the plant with carbon-13 can be very costly. However, the apparent lack of a measurable isotope effect of carbon-13 and the methodological advantage of a uniformly labeled molecule are advantages of fully labeling a plant with carbon-13. In our assessment, the methodological advantages of fully labeling kale with carbon-13 surpassed the disadvantages of cost and requirement of specialized equipment. Previous use of atmospheric labeling of plants includes assessment of carbon assimilation in photosynthesis (*44*−*48*) and investigation of atmospheric labeling as a means of producing carbon-13-labeled starch, glucose, fructose, and sucrose in kilogram quantities from tobacco leaves (*49*).

Kale was chosen as the initial crop for labeling for several reasons. Kale is rich in nutrients (high in not only β-carotene, lutein, and vitamin K_1 but also folate, ascorbate, and flavonoids), kale has a fairly short growth cycle (2−3 weeks), and kale has a favorable harvest index (its high edible-to-inedible ratio minimizes the loss of expensive isotopic label in inedible portions of the plant). Our initial nutrient focus is lutein because of its potential to prevent macular degeneration, β-carotene because of its role in providing vitamin A, and phylloquinone for its role in blood clotting and cardiovascular disease risk. Other researchers have used stable isotopes to study bioavailability of these compounds, including (i) purified, deuterium-labeled β-carotene (*13*, *14*, *16*−*18*), (ii) purified, carbon-13-labeled β-carotene (*12*, *15*, *18*, *20*, *22*), (iii) purified, carbon-13-labeled lutein (*17*), and (iv) deuterium-labeled phylloquinone in broccoli (*23*). The use of stable isotopes for bioavailability studies of carotenoids was recently reviewed by van Lieshout et al. (*50*). To our knowledge, this is the first study to use atmospheric labeling to produce carbon-13-labeled plants for in situ nutrient bioavailability studies.

New analytical methodology was required to complete the pilot study. Previous methods had been published for analysis of labeled lutein in plasma (*17*), of labeled β-carotene in plasma (*24*−*28*), of labeled retinol in plasma (*28*−*31*), and of labeled phylloquinone in plasma (*23*, *32*). Our current instrument capabilities combined with efficiency requirements led us to develop a new method for analysis of these nutrients using LC-APCI-MS. The LC-MS method was modified from Wang et al. (*27*) to allow for simultaneous selective detection of ^{13}C-labeled and unlabeled carotenoids, retinol, and phylloquinone. We assumed that isotopic distribution in plasma would be the same as in kale and therefore used the same base peaks for quantification. However, there is no retinol in kale, so we used the completely labeled retinol molecule ($^{13}C_{20}$) with *m*/*z* 289 for quantification.

β-Carotene elutes from the HPLC column at 23.4 min with no interference in the DAD. Unfortunately, use of this chromatographic method for plasma produces a coeluting mass spectral interference at *m*/*z* 577 (thus impeding quantification of $^{13}C_{40}$-β-carotene). Only minor modifications of the chromatographic system were attempted in order to preserve the unobstructed quantification of the other analytes. When this proved unsuccessful, SPE was used to separate the β-carotene from the unknown metabolite. Recovery studies were carried out and showed that 80% of the β-carotene was recovered from the SPE cartridges.

Lutein, β-carotene, and phylloquinone contents of the kale were lower than average reported values (*51*−*55*). It is not clear to what extent these results were related to carbon isotope, to kale variety, or to growth conditions unique to the labeling procedures. Nonetheless, the content of these nutrients was sufficient to provide a detectable plasma response after consumption of the vegetable dose.

If the subject is assumed to have 45 mL of plasma per kg of body weight (*56*), then one can calculate the portion of the dose of each nutrient that is found in plasma at a given time point. This calculation showed that the peak lutein concentration accounted for 3% (0.88 μmol) of the dose, the peak β-carotene concentration accounted for 1% (0.24 μmol) of the β-carotene dose, the peak retinol concentration accounted for 1% (0.38 μmol) of the β-carotene dose (assuming that 2 mol of retinol is equivalent to 1 mol of β-carotene), and the peak phylloquinone concentration accounted for 7% (11.5 nmol) of the dose. Extrapolating this information to relative bioavailabilities must be done with caution, since these peak values are impacted not only by absorption but also by elimination rates. With respect to the carotenoids, one might expect a greater difference in peak

values considering the difference in levels of enrichment of the endogenous pool with labeled nutrient (^{13}C-lutein peaking at 82% of the endogenous lutein level, β-carotene peaking at 21% of the endogenous β-carotene level, and retinol peaking at 3% of the endogenous retinol level). Because these data represent only one subject, one must be cautious about not drawing bioavailability conclusions from these results, especially given the variable plasma response observed previously for carotenoids. With respect to β-carotene, studies have consistently shown tremendous variation in plasma response after ingestion (*57*−*61*). Furthermore, Bowen et al. (*62*) saw a variable plasma response for several carotenoids in subjects receiving six fruits and vegetables per day for several weeks, showing that this phenomenon is not exclusive to β-carotene. Extension of these protocols to more subjects and in-depth kinetic analysis (*13*, *14*) of our data will provide more information about relative bioavailabilites of these nutrients.

In summary, this method of labeling green, leafy vegetables with ^{13}C for use in a clinical feeding study, followed by quantitation of labeled nutrients in plasma by LC-APCI-MS, was successful for producing clearly defined kinetic curves for ^{13}C-β-carotene, ^{13}C-retinol, ^{13}C-lutein, and ^{13}C-phylloquinone.

ABBREVIATIONS USED

APCI, atmospheric pressure chemical ionization; BHT, butylated-hydroxytoluene; DAD, diode array detector; EtOH, ethanol; GC-MS, gas chromatography−mass spectrometry; LC-MS, liquid chromatography−mass spectrometry; MeOH, methanol; MTBE, methyl tertiary-butyl ether; *m*/*z*, mass-to-charge ratio; NIST, National Institute of Standards and Technology; SIM, selected ion monitoring; SPE, solid phase extraction.

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