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Short communication

Use of high-precision gas isotope ratio mass spectrometry to determine natural abundance ^{13}C in lutein isolated from C_3 and C_4 plant sources

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Abstract

A method was developed for high-precision stable carbon isotope ratio analysis of lutein isolated from a C_3 (marigold flower) and a C_4 (corn gluten meal) plant source using gas chromatography–combustion interfaced isotope ratio mass spectrometry. The natural abundance of ^{13}C (expressed as $\delta^{13}C$ versus the international standard, Pee Dee Belemnite, in per mil units, denoted ‰) in lutein isolated from marigold flower and corn gluten meal was determined to be -29.90 ± 0.20‰ and -19.77 ± 0.27‰ (mean±S.D.), respectively. The high precision of gas isotope ratio mass spectrometry is potentially applicable to detect differences of isotopic composition of lutein in the blood, tissues, or excreta of animal models or humans that result from differences in the natural abundance of ^{13}C in C_3 and C_4 plant foods. © 1998 Elsevier Science B.V.

Keywords: Lutein; Carotenoids

1. Introduction

Gas chromatography–combustion interfaced isotope ratio mass spectrometry (GC–C-IR-MS) enables direct stable carbon isotope ratio analysis of individual, volatile organic compounds in chemically complex samples with high precision [1]. Application of this technique to analysis of carotenoids, such as lutein (β,ϵ-carotene-3,3′-diol), requires hydrogenation to thermally stable perhydro analogs because of the instability of the conjugated polyene chains at high temperatures [2].

Lutein is the predominant carotenoid in yellow corn and dark-green leafy vegetables [3] and accumulates primarily from plant foods in the sera and tissues of humans [4,5]. Lutein and zeaxanthin (β,β-carotene-3,3′-diol), isomeric dihydroxycarotenoids (Fig. 1), are selectively deposited from the blood in the macular lutea of the human retina [6,7]. This area of the retina is exposed to focused radiant energy of wavelengths within the absorption spectra of lutein and zeaxanthin, and these macular pigments may function in photoprotection [8]. In the Eye Disease Case-Control Study [9], high intakes of dark-green leafy vegetables and corresponding high intakes of lutein and zeaxanthin were particularly associated with substantially lower risk of age-related macular degeneration, which is the leading cause of irreversible blindness in older adults [10]. This epidemiological observation, dietary depletion of macular pigment in non-human primates [11], and recent lutein supplementation trials in humans [12,13] indicate that the density of the macular pigment is modifiable by dietary manipulation. Thus there is a need to evaluate the extent to which lutein is

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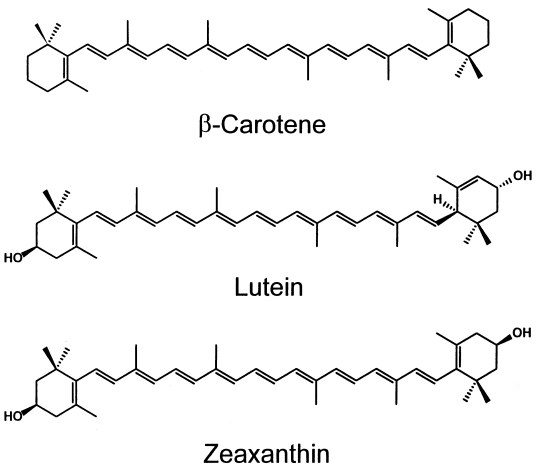

Fig. 1. Chemical structures of β-carotene, lutein and zeaxanthin.

absorbed in the small intestine after consumption of lutein-rich plant foods.

Gas IR-MS provides the high precision needed to detect differences of natural abundance of ^{13}C such as differences of isotopic composition of C_3 relative to C_4 photosynthetic plants [14]. The C_3 and C_4 photosynthetic pathways differ in the isotopic discrimination of the initial carboxylating enzyme that incorporates CO_2 from the atmosphere [15]. Ribulose bisphosphate carboxylase in C_3 photosynthesis discriminates against atmospheric $^{13}CO_2$ to a greater degree than phosphoenol pyruvate carboxylase in C_4 photosynthesis. As a result of the isotopic fractionation by ribulose bisphosphate carboxylase and of differential CO_2 diffusion rates through stromata, the ^{13}C content of C_4 plants is higher than that of C_3 plants [16]. Carbon isotope fractionation in plants is potentially useful for studies of the bioavailability and metabolism of ^{13}C-enriched lutein derived from C_4 plant foods in animal models or humans. The objective of the current study was to use GC–C-IR-MS to determine the stable carbon isotope ratios (expressed as $\delta^{13}C$ values) of lutein isolated from a C_3 (marigold flower) and a C_4 (corn gluten meal) plant source.

2. Experimental

2.1. Extraction and purification of lutein

A commercially available standard of lutein extracted from marigold flowers (reagent grade lutein, 95% purity, generously donated by Kemin Industries, Des Moines, IA, USA) was used without further purification. Lutein in corn gluten meal was extracted using procedures adapted from Livingston et al. [17]. The corn gluten extract was prepared by soaking 4.5 g of a standard commercial corn meal in 19 ml of hexane–acetone–ethanol–water (6:6:6:1, v/v) for 24 h with agitation at room temperature. The mixture was then vortexed and centrifuged (500 *g*, 10 min). The supernatant was collected, and the corn meal was washed twice with additional solvent (6 ml per wash), vortexed, and centrifuged. The combined supernatants were concentrated to one-third the original volume under nitrogen, diluted with 5 ml of diethyl ether, and washed with 2 ml of water to remove ethanol and acetone. The mixture was vortexed, centrifuged (250 *g*, 1 min), and the upper diethyl ether layer was removed. The lower aqueous layer was extracted twice with 3 ml of diethyl ether, and the combined diethyl ether layers were evaporated to dryness under nitrogen. The residue was reconstituted with 100 μl of methanol–tetrahydrofuran (THF) (70:30, v/v) and 50 μl were injected into the high-performance liquid chromatography (HPLC) system. Procedures were performed under dim or yellow light to minimize photodegradation of lutein.

Lutein was purified from the corn meal extract by HPLC using the following equipment (Waters, Milford, MA, USA): a 717Plus autosampler with temperature control set to 5°C, two 510 solvent delivery systems, and a 996 photodiode array detector. The system was operated with Millennium 2010 Chromatography Manager software. The lutein fraction was collected using a 5-μm C_{30} 250×4.6 mm analytical column (Carotenoid Column, YMC, Wilmington, NC, USA) [18] and a linear gradient (100:0 methanol–tetrahydrofuran to 0:100 methanol–THF over 20 min; 1 ml/min) to provide baseline resolution of lutein and the structurally isomeric carotenoid, zeaxanthin. Solvents were HPLC grade; methanol was purchased from Fisher Scientific (Chicago, IL, USA) and THF (OmniSolv) was purchased from Baxter Diagnostics (McGaw Park, IL, USA). The mobile phase was filtered (Nylon-66 filter, 0.2 μm, Rainin Instruments, Woburn, MA, USA). The purity of the isolated lutein was supported by comparison of spectral data with that of the commercial lutein

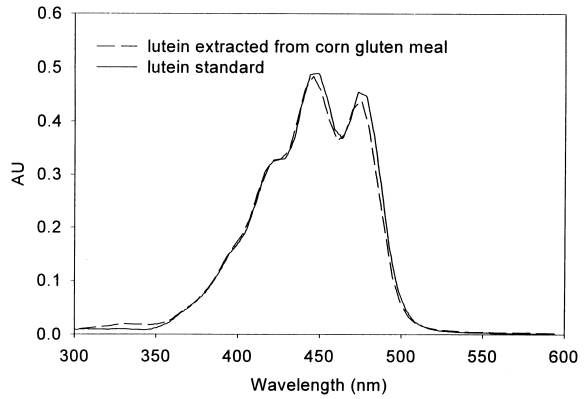

Fig. 2. Overlay of UV–visible absorption spectra of lutein extracted from corn gluten meal and of a commercial lutein standard from marigold flowers (reagent grade lutein, 95% purity, Kemin) normalized to the same absorbance scale. The spectra were recorded during reversed-phase HPLC analysis using a 5 μm C_{30} 250×4.6 mm analytical column (carotenoid column, YMC), a linear gradient (100:0 methanol–THF to 0:100 methanol–THF over 20 min; 1 ml/min), and a Waters 996 photodiode array detector (Waters).

standard extracted from marigold flower (reagent grade lutein, 95% purity) (Fig. 2).

2.2. Hydrogenation

Purified lutein was dissolved in chloroform and hydrogenated to prevent thermal decomposition during GC separation prior to MS analysis. Platinum(IV) oxide (Alfa Aesar, Ward Hill, MA, USA) catalyst was added to 1 ml glacial acetic acid–chloroform (1:1, v/v) and maintained under positive hydrogen gas pressure for 40 min prior to addition of 0.02 ml of lutein in chloroform (100 μg/ml). The reaction mixture was maintained under hydrogen gas overnight with stirring at 60°C. The mixture was washed twice with 1.0 ml of water to remove acid and catalyst and evaporated to dryness under nitrogen. The residue was reconstituted in 10 μl of hexane; aliquots of 2 μl were injected for GC–MS and GC–C-IR-MS analyses.

2.3. Mass spectrometry

A Varian (Walnut Creek, CA, USA) GC 3400 chromatograph interfaced to a Finnigan (San Jose, CA, USA) TSQ 700 triple quadrupole mass spectrometer (GC–MS) in electron ionization mode (70 eV) was employed for identification of hydrogenated lutein. A 10 m×0.25 mm I.D. (0.25 μm film thickness) DB-1 (J&W Scientific, Folsom, CA, USA) fused-silica capillary column with on-column injector was used with ultrapure helium as carrier gas at a flow-rate of 40 cm/s. The temperature program proceeded from 50°C followed by a gradient of 30°C/min to 150°C followed by a gradient of 15°C/min to 325°C and a 20 min hold at 325°C. The interface temperature was 325°C and the source temperature was 150°C. Mass spectra were acquired over the range m/z 35–650 in 0.75 s.

Lutein isolated from each plant source was hydrogenated and injected into a 5890A Hewlett-Packard (Wilmington, DE, USA) gas chromatograph (same operating conditions as above) fitted with a Fisons/VG Isotech Isochrom gas chromatograph–combustion interface to the Fisons/VG Isotech (currently Micromass UK, Manchester, UK) Optima isotope ratio mass spectrometer. To evaluate the potential for isotopic fractionation during hydrogenation, the $^{13}C/^{12}C$ ratio of lutein isolated from marigold flowers was also analyzed without hydrogenation using a NA1500 elemental analyzer (EA) (CE Elantech, Lakewood, NJ, USA) interfaced to the Optima isotope ratio mass spectrometer. Lutein was quantitatively combusted to CO_2 and H_2O; H_2O vapor was removed by a chemical trap, and the CO_2 was further purified by GC on a Poropak QS column (2 m×4 mm I.D.) at 60°C and admitted into the Optima isotope ratio mass spectrometer [19]. Carbon isotope ratio measurements are expressed in delta (δ), per mil (‰) units, as follows:

$$\delta^{13}\mathrm{C},\text{‰} = \left(\frac{R_{\mathrm{sample}}}{R_{\mathrm{standard}}} - 1\right) \times 1000$$

where $R = {}^{13}C/{}^{12}C$. The isotope ratio of each sample was determined by comparison with a working standard of CO_2 gas, which, in turn, was calibrated against CO_2 generated from international limestone standards and had an isotope ratio of −31.91‰ relative to Pee Dee Belemnite (PDB). The international standard for carbon is a sample of calcium carbonate obtained from the Pee Dee formation in South Carolina with an accepted value of $R_{\mathrm{PDB}} = 0.0112372 \pm 0.000009$ [20].

3. Results

The hydrogenation of lutein produced perhydro-β-carotene as the major product which was completely resolved by GC from higher-molecular-mass secondary products that co-eluted as a single broad peak (Fig. 3). The identity of the perhydro-β-carotene was based on comparison of GC retention time with that of perhydro-β-carotene produced by hydrogenation of a synthetic β-carotene standard (>97% purity, Fluka, Ronkonkoma, NY, USA) (Fig. 3), and was further confirmed by GC–MS (Fig. 4). The mass spectra of hydrogenated β-carotene and of the major product of lutein hydrogenation both showed a molecular ion at m/z 558.6; 558.6 is the molecular mass of perhydro-β-carotene. The co-eluting secondary products of lutein hydrogenation showed molecular ions at m/z 572.6 and m/z 574.7 (data not shown). The peak that corresponded to the perhydro-β-carotene product of lutein hydrogenation was selected for carbon stable isotope ratio measurement

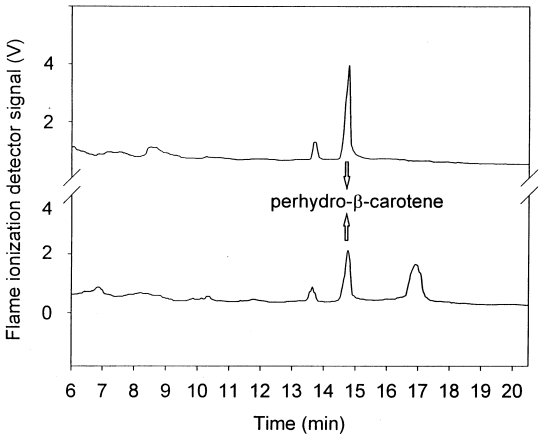

Fig. 3. Gas chromatograms of hydrogenated synthetic β-carotene standard (>97% purity, Fluka) (upper trace) and hydrogenated lutein standard from marigold flower (reagent grade lutein, 95% purity, Kemin) (lower trace). The unidentified minor peak that eluted before perhydro-β-carotene was also detected after hydrogenation of a reagent blank in the absence of carotenoid. A 10 m×0.25 mm I.D. (0.25 μm film thickness) DB-1 (J&W Scientific) fused-silica capillary column with on-column injector was used with ultrapure helium as carrier gas at a flow-rate of 40 cm/s. The temperature program proceeded from 50°C followed by a gradient of 30°C/min to 150°C followed by a gradient of 15°C/min to 325°C and a 20 min hold at 325°C. The identity of the perhydro-β-carotene peak was confirmed by GC–MS.

by GC–C-IR-MS; the broad peak shape of the co-eluting secondary products was not conducive to high-precision stable carbon isotope ratio analysis. A representative GC–C-IR-MS chromatogram of hydrogenated commercial lutein standard extracted from marigold flower (reagent grade lutein, Kemin Industries) is shown in Fig. 5.

To determine whether isotopic fractionation occurred during hydrogenation, the carbon isotope ratio of commercial lutein standard extracted from marigold flower (95% purity) was measured by EA–IR-MS without hydrogenation. The carbon isotope ratio ($\delta^{13}C$) of lutein from marigold flower was -30.82 ± 0.10‰ (mean±S.D., $n=4$). The $\delta^{13}C$ value of the same commercial lutein standard from marigold flower was also determined using GC–C-IR-MS after hydrogenation. The $\delta^{13}C$ value of the perhydro-β-carotene peak was -29.90 ± 0.20‰ ($n=3$). A comparison of the $\delta^{13}C$ values indicates that no appreciable isotopic fractionation occurred during hydrogenation of lutein.

Lutein isolated from corn gluten meal, a C_4 plant source, was also hydrogenated and analyzed by GC–C-IR-MS. The $\delta^{13}C$ value was -19.77 ± 0.27‰ ($n=7$). This value was significantly different from the $\delta^{13}C$ value of -29.90 ± 0.20‰ determined for lutein isolated from marigold flower, a C_3 plant source. These data are consistent with the greater ^{13}C content of C_4 plants.

4. Discussion

The conjugated polyene system of carotenoids is thermally labile so that hydrogenation is necessary prior to GC analysis. Hydrogenation of the hydrocarbon β-carotene with platinum as catalyst is complete and produces a single product (Fig. 3). In contrast, catalytic hydrogenation of the dihydroxy-carotenoid lutein produces the major product perhydro-β-carotene (m/z 558.6) and secondary products. The secondary products have mass spectra that are consistent with saturated (m/z 574.7) and monounsaturated (m/z 572.6) forms of anhydrolutein (the monohydroxy derivative), a product which most likely results from the favored acid-catalyzed dehydration of the allylic hydroxyl group of lutein [21]. Acetylation, methylation, or silylation of the hy-

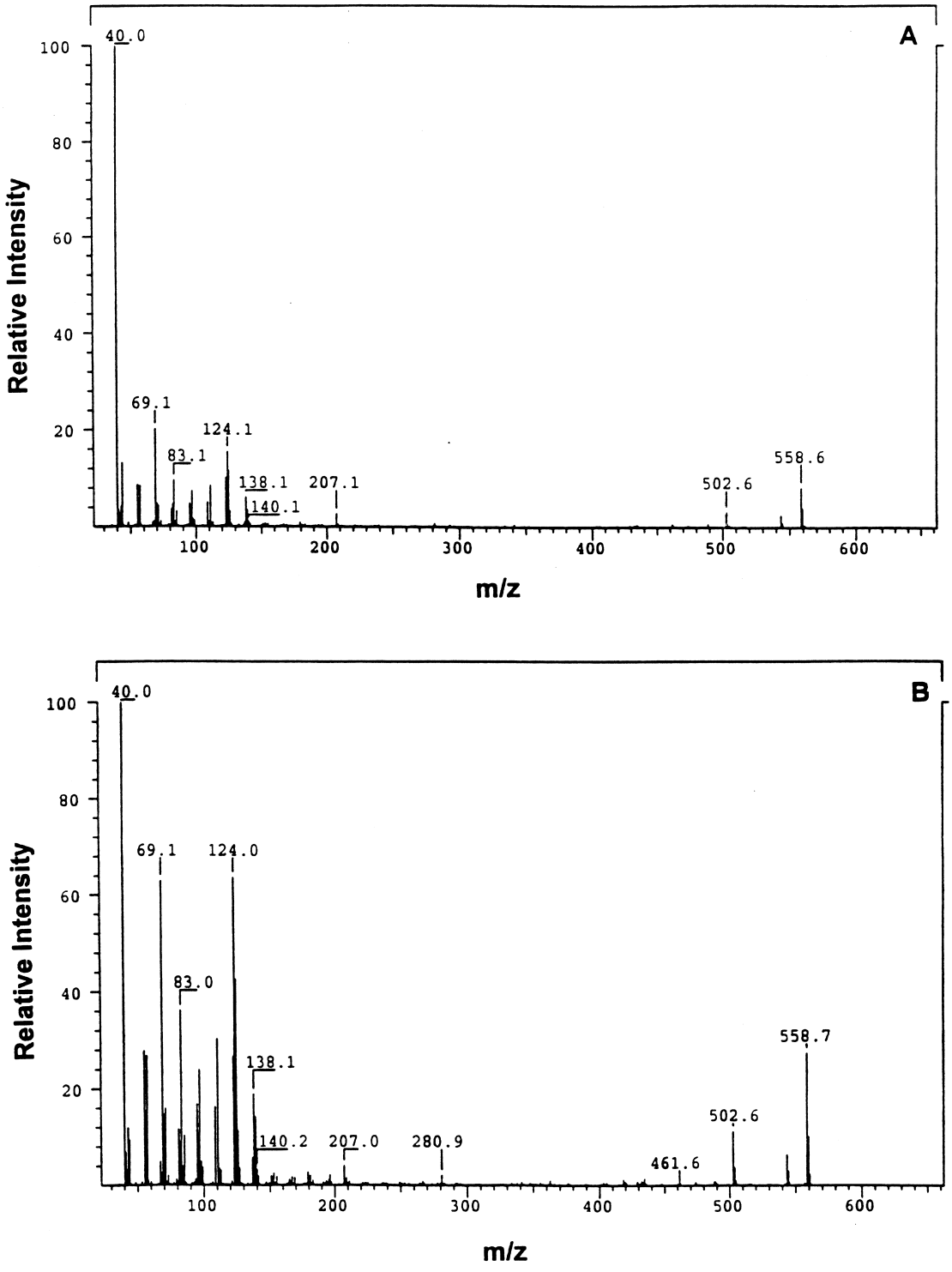

Fig. 4. Electron ionization (70 eV) mass spectra showing similar fragmentation of the product of β-carotene hydrogenation (A) and of the major product of lutein hydrogenation (B). Both produced the perhydro-β-carotene molecular ion at m/z 558.6.

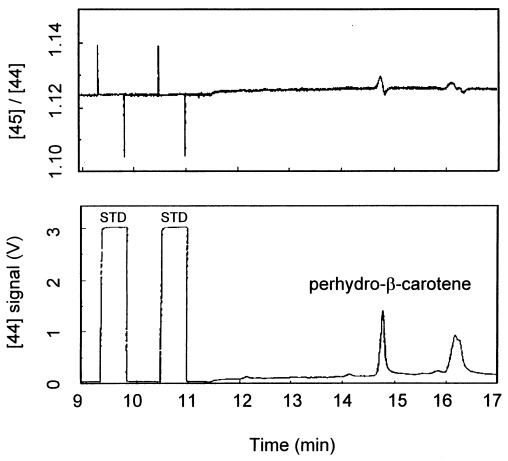

Fig. 5. GC–C-IR-MS plot of hydrogenated commercial lutein standard from marigold flower (reagent grade lutein, 95% purity, Kemin). The lower trace is the signal from the m/z 44 detector; the upper trace is the ratio of the m/z 45 to m/z 44 signals. The square pulses labeled ''STD'' are signal due to calibrated CO_2 gas admitted from one of the dual-inlet volumes. The GC operating conditions are described in Fig. 3.

droxyl groups of lutein after hydrogenation produced perhydro-β-carotene as a side-product; this is consistent with the derivatization of other hydroxycarotenoids [22]. Derivatization of the hydroxyl groups before hydrogenation is associated with losses of added acetyl or trimethylsilyl groups, possibly as a result of hydrogenolysis [22]. In addition, silylation adds carbon atoms which can reduce the $\delta^{13}C$ value of GC–C-IR-MS samples, whereas acetylation can result in carbon isotopic fractionation [23]. Thus chemical derivatization of the hydroxyl groups of lutein before or after hydrogenation does not appear to be advantageous relative to hydrogenation alone.

To calibrate isotopic measurements of the major product of lutein hydrogenation, perhydro-β-carotene, we introduced pulses of isotopically calibrated CO_2 gas through the dual inlet directly into the ion source of the IR-MS system. A limitation of conventional calibration using CO_2 standard is the inability to correct for imprecision arising from the physical conditions to which the analyte is subjected during passage through the gas chromatograph and combustion interface. An alternative approach is to use a noninterfering isotopically calibrated internal standard added directly to the analysis mixture [24]. External CO_2 standard was used in our study to allow free positioning of the CO_2 reference peaks within the gas chromatogram and preclude coelution with minor sample components present in the lutein isolated from corn gluten meal (data not shown); coelution of standards with sample components is associated with systematic errors in excess of 1‰ even when overlaps appear to be minor [25]. In a controlled comparison, introduction of CO_2 reference standard from a conventional inlet during GC–C-IR-MS resulted in accuracy and precision comparable to that obtained with coinjection of internal standard [25].

Our data indicate that hydrogenation of lutein does not result in appreciable carbon isotopic fractionation, and therefore the perhydro-β-carotene product is suitable for GC–C-IR-MS analysis. Using commercial lutein extracted from marigold flowers (95% purity) as starting material, the $\delta^{13}C$ value of perhydro-β-carotene produced by hydrogenation was determined to be -29.90 ± 0.20‰ using GC–C-IR-MS. This compares well with the $\delta^{13}C$ value of -30.82 ± 0.10‰ for the same lutein standard measured without hydrogenation using EA–IR-MS. The difference is within the range of analytical variability associated with different methods of sample preparation and combustion to CO_2. For example, the limits of agreement of measured and accepted $\delta^{13}_{PDB}C$ values for isotope standards analyzed by the NA1500 elemental analyzer coupled to a gas isotope ratio mass spectrometer were reported to be between -1.01 and $+1.17$‰ [19].

The high precision of GC–C-IR-MS was used to detect differences of natural abundance ^{13}C in the dihydroxycarotenoid lutein isolated from a C_3 plant source, marigold flower, and from a C_4 plant source, corn gluten meal. Corn and other tropical grasses, such as sorghum, and sugar cane, use the C_4 photosynthetic pathway and are relatively enriched in ^{13}C. These C_4 plants have $\delta^{13}C$ values of -10 to -19‰ [26]. Previous mass spectrometric analyses of corn cobs and kernels showed $\delta^{13}C$ values of -10 to -12‰ [27]. In our study, the measured $\delta^{13}C$ value of lutein isolated from corn gluten meal was -19.77 ± 0.27‰. In both C_3 and C_4 plants, lipids and carotenoids are reported to be enriched by approximately 5‰ in ^{12}C relative to total carbon [28]. Thus our measured $\delta^{13}C$ value for lutein

isolated from corn gluten meal is in good agreement with that predicted by the existing literature. Marigolds and most higher plants use the C_3 photosynthetic pathway and have $\delta^{13}C$ values of -24 to -34‰ [29]. Even with the consideration that the reported carbon isotopic composition of carotenoids is lighter by approximately 5‰, there is good agreement with our measured $\delta^{13}C$ value of -29.90 ± 0.20‰ for lutein isolated from marigold flower.

There is no large isotopic fractionation associated with the incorporation of carbon from a diet into an animal [30], and thus natural-abundance isotope ratios in animals are determined to a large extent by the types of foods (in terms of method of carbon fixation and number of trophic levels) consumed over a period of weeks or months [31]. Because carotenoids cannot be synthesized by mammals and thus are incorporated directly from the diet [32], lutein in mammalian blood and tissues would be expected to have $\delta^{13}C$ values identical to those of lutein in the diet provided that there is no fractionation during incorporation or metabolism [30]. The human diet consists primarily of foods derived from C_3 plants. Of the five major carotenoids in human plasma (α-carotene, β-carotene, β-cryptoxanthin, lutein and lycopene), lutein is unique in that prominent dietary sources include both C_3 plant foods, such as spinach and other dark-green leafy vegetables, and also the C_4 plant food, corn [3]. Thus the natural ^{13}C enrichment of corn and corn products is potentially applicable for studies of the bioavailability of lutein in plant foods fed to animal models or humans.

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