

Carrot solution culture bioproduction of uniformly labeled ^{13}C -lutein and *in vivo* dosing in non-human primates

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Abstract

Lutein is a xanthophyll abundant in nature and most commonly present in the human diet through consumption of leafy green vegetables. With zeaxanthin and *meso*-zeaxanthin, lutein is a component of the macular pigment of the retina, where it protects against photooxidation and age-related macular degeneration. Recent studies have suggested that lutein may positively impact cognition throughout the lifespan, but outside of the retina, the deposition, metabolism, and function(s) of lutein are poorly understood. Using a novel botanical cell culture system (*Daucus carota*), the present study aimed to produce a stable isotope lutein tracer for use in future investigations of dietary lutein distribution and metabolism. Carrot cultivars were initiated into liquid solution culture, lutein production conditions optimized, and uniformly labeled ^{13}C -glucose was provided as the sole media carbon source for four serial growth cycles. Lutein yield was $2.58 \pm 0.24 \mu\text{g/g}$, and mass spectrometry confirmed high enrichment of ^{13}C : 64.9% of lutein was uniformly labeled and 100% of lutein was labeled on at least 37 of 40 possible carbons. Purification of carrot extracts yielded a lutein dose of 1.92 mg with $96.0 \pm 0.60\%$ purity. ^{13}C -lutein signals were detectable in hepatic extracts of an adult rhesus macaque monkey (*Macaca mulatta*) dosed with ^{13}C -lutein, but not in hepatic samples collected from control animals. This novel botanical biofactory approach can be used to produce sufficient quantities of highly enriched and pure ^{13}C -lutein doses for use in tracer studies investigating lutein distribution, metabolism, and function.

Keywords: Lutein, phytochemicals, nutrition, isotopic tracer, mass spectrometry, non-human primate

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Introduction

Lutein is a 40-carbon hydroxylated carotenoid (Figure 1), abundant throughout nature, which accounts for up to 50% of total carotenoid content of higher plants.¹ In plants, it serves several important roles: light harvesting through transfer of excitation energy to chlorophyll, stabilization of proteins within the light harvesting complex, and free radical quenching.² Lutein-rich green leafy vegetables are the major dietary source of this carotenoid for Americans,^{3,4} and results from the National Health and Examination Survey 2003–2004 indicate that average intake is highest in older adults ($1008 \pm 96 \mu\text{g}$ lutein/day) and those of non-Hispanic black ethnicity ($1118 \pm 111 \mu\text{g}$ lutein/day).⁴ After ingestion, lutein is absorbed and distributed to a number of tissues, most notably being the eye, where it comprises a major portion of the retinal macular pigment.^{5,6} In the retina, lutein, along with zeaxanthin and

the lutein metabolite *meso*-zeaxanthin,^{7,8} are thought to protect against age-related macular degeneration, a major cause of blindness in the USA.^{9–11} Much work has been done on the role of lutein in the preservation of retinal health, but recent research also has suggested a role for this carotenoid in the brain. In a study by Vishwanathan et al., brain carotenoid concentrations in decedent infants reflected a preferential accumulation of lutein compared to other carotenoids, despite the fact that lutein was not the predominant carotenoid in the diets of pregnant mothers, breastfeeding mothers, or infants.¹² This relationship holds true at the other extreme of the human lifespan: while the serum carotenoid profile of centenarians reflected a typical diet (high concentrations of β -carotene and lycopene, lower levels of lutein), brain carotenoids revealed a preferential accumulation of lutein.¹³ Moreover, post-mortem brain lutein levels in these centenarians correlated with

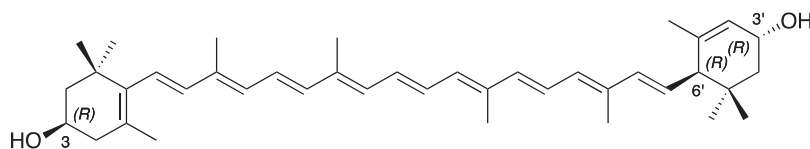


Figure 1 Chemical structure of lutein [(3*R*,3'*R*,6'*R*)- β,ϵ -Carotene-3,3'-diol]

pre-mortem cognitive performance,¹³ and a randomized, double blind, placebo-controlled trial found that four months of lutein supplementation improved cognition in elderly women.¹⁴ While hypotheses have been put forward, a mechanism of action for these effects has yet to be resolved. Work in our group,¹⁵ as well as by others, is currently proceeding towards this goal.

Elucidation of dietary bioactives' mechanism(s) of action *in vivo* is aided greatly by the ability to follow ingested compounds through the various steps of absorption, tissue deposition, and metabolism in a human subject or an appropriate animal model. Isotopically labeled tracers have been of great utility in studies of retinol and β -carotene metabolism,^{16,17} indeed, we have already used this technique to investigate the tissue deposition and metabolism of lycopene and phytoene.^{18,19} Total chemical synthesis of lutein has been previously described,²⁰ making isotopic labeling through this approach technically possible; however, the tracer masses required for typical feeding studies prevent this approach from being economically feasible for most investigators. Several investigators have used microbial^{21–23} or botanical^{24–27} biofactory approaches for the production of isotopically labeled lutein tracers. Similarly, we have experience in both microbial²⁸ and botanical^{29–32} approaches for the production of isotopically labeled carotenoids, but have not previously applied these to lutein tracer production. Botanical biofactory methods are reviewed in greater detail elsewhere.¹⁵

Efficiency of molecular labeling with stable isotopes can be validated using mass spectrometry (MS).³³ Labeling of molecules with stable isotopes results in changes in their molecular masses and isotopic signatures, thus making them amenable to unambiguous detection and characterization.³³ Not surprisingly, the history of studies of stable isotope tracers and development of MS methods are closely linked.³⁴ MS multiplexed analysis allows not only the detection of target labeled compounds, but also the determination of degree of isotopic labeling and ratios of completely and incompletely labeled molecules.^{35,36} Moreover, such analyses reveal isotopic patterns of both endogenously present and exogenous metabolites of targeted molecules.³⁷ Therefore, the combination of targeted quantitative MS analysis and semi-quantitative MS profiling can be used for pharmacokinetics and pharmacodynamics of ¹³C-lutein and other carotenoids in biological specimens. MS has been widely used in detection of isotopically labeled carotenoids, including ¹³C-lutein in human plasma.^{22–24,26}

This study sought to establish a novel botanical model for the stable isotopic labeling of lutein, optimize lutein production *in vitro*, and validate MS methods for the

identification of labeled lutein in plant cell culture and hepatic tissue of a dosed animal. Future work, which is already underway, will harness the produced lutein tracer for studies of lutein distribution and metabolism in the non-human primate brain, with the goal of identifying mechanisms by which lutein may influence cognition in primates, including humans.

Materials and methods

Chemicals

HPLC-grade acetonitrile, diethyl ether, dioxane, ethyl acetate, methanol, triethylamine, chloroform, and water were purchased from Fisher Scientific (Fair Lawn, NJ, USA), as were potassium hydroxide and sodium chloride. Butylated hydroxytoluene, sodium chloride, and HPLC-grade 2-propanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethanol (200 proof) was purchased from Decon Labs (King of Prussia, PA, USA). 2,4-dichlorophenoxyacetic acid was purchased from Sigma-Aldrich and cell culture media (Murashige & Skoog, Gamborg B5) were purchased from PhytoTechnology Laboratories (Overland Park, KS, USA). Uniformly labeled ¹³C-glucose was purchased from Cambridge Isotope Laboratories (Tewksbury, MA, USA). Deuterated GABA (4-Aminobutyric-2,2-d₂ acid, 98 atom % D, Cat. no. D-1731) was obtained from C/D/N Isotopes (Pointe-Claire, Canada).

Seed disinfestation, germination, cell culture, and harvest

Seeds of yellow, purple, and red *Daucus carota* (carrot) cultivars Amarillo, Jaune Obtuse du Doubs (Bakercreek Heirloom Seeds, Mansfield, MO), Yellowstone, Yellow Solaris (Irish Eyes Garden Seeds, Thorp, WA, USA), Purple Dragon, Purple Sun, and Atomic Red (John Scheepers Kitchen Garden Seeds, Bantam, CT, USA) were washed with deionized water for 20 min followed by a 1-min immersion in 70% ethanol, and then soaked in a solution of 1% sodium hypochlorite solution and dilute detergent for 15 min. Seeds were rinsed three times in sterilized deionized water, explanted onto 15 mL germination medium consisting of half-strength Gamborg B5 (B5) salts and vitamins, myoinositol (0.1 g/L), sucrose (30 g/L), and agar (6 g/L) in capped 25 × 150 mm culture tubes, and placed under approximately 30–40 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of continuous cool white fluorescent lighting at a temperature of 25 ± 2°C. After 13 to 17 days of germination, the resulting seedlings were divided into root, hypocotyl, cotyledon, or true leaf sections and transferred to tubes of callus induction medium similar to the seedling germination medium, with the exception of the use of full strength B5 salts and the

addition of 1 mL/L 2,4-dichlorophenoxyacetic acid. A total of 66 callus lines were generated in this manner. Explants were transferred to fresh agar-solidified callus induction medium every 28 days and approximately 1 cm callus sections were excised from mother tissue when sufficient growth had occurred. As growth rate increased, cubes containing 40 mL medium were used as growth vessels containing 4 callus clumps per cube.

After several months, when sufficient friable callus was available, solution cultures were induced by transferring approximately 2 g of callus to 40 mL of liquid medium, composed of the same components as callus initiation medium, within a 125 mL Erlenmeyer flask. Flasks were placed in a rotary shaker at 150 r/min at a temperature of $25 \pm 2^\circ\text{C}$ with approximately $40 \mu\text{mol m}^{-2} \text{s}^{-1}$ of continuous cool white fluorescent lighting. To maintain the cultures, 5 mL of suspended cells were transferred to fresh medium at 14-day intervals. Unless otherwise specified, these conditions were employed in all experiments.

Harvesting of callus and cells was conducted at the end of growth cycles of 28 days for solid callus and 14 days for solution cultures. Mass data for callus grown on solid media were not collected due to inaccuracy in separating callus tissue and gelled media. Cell mass data for solution cultures was collected by pouring into pre-weighed 50 mL centrifuge tubes, centrifuging for 5 min at 2500 r/min, pouring off excess liquid, reweighing, and calculating mass. All callus samples were frozen at -80°C until extraction.

Culture optimization

Basal salt and carbon source. Previously, both B5 and Murashige and Skoog (M&S) media have been used to successfully grow carrot solution cultures. These media utilize sucrose as a carbon source, but due to the lack of economical sources of ¹³C-sucrose, it was necessary to determine the suitability of glucose as a carbon source for the subsequent labeling protocol. A $2 \times 2 \times 2$ factorial experiment was arranged such that two replicates of established solution cultures of an Amarillo root cell line and a Jaune Obtuse du Doubs hypocotyl cell line were supplied with either glucose or sucrose as the sole carbon source in each of the B5 or M&S basal salt culture media conditions. Cells were allowed to grow for 14 days and then were harvested for lutein extraction and HPLC analysis.

Light and light intensity preference. To determine the effect of light exposure and intensity on the production of lutein, cells were grown in duplicate under $40 \mu\text{mol} \bullet \text{m}^{-2} \bullet \text{s}^{-1}$ or $80 \mu\text{mol} \bullet \text{m}^{-2} \bullet \text{s}^{-1}$ of cool white continuous light, or with light exclusion provided by foil covering. Cultures were harvested for lutein extraction and HPLC analysis after 14 days of treatment.

Flask size. The effect of flask size on lutein production was tested in 125 mL and 250 mL Erlenmeyer flasks. For both flask sizes, final culture volume was 36% of the total flask capacity, consisting of 32% fresh media and 4% cell inoculum (40 mL fresh media + 5 mL inoculum for 125 mL flasks; 80 mL fresh media + 10 mL inoculum for 250 mL flasks).

All cultures were grown in duplicate under $80 \mu\text{mol} \bullet \text{m}^{-2} \bullet \text{s}^{-1}$ of cool white continuous light for 14 days, after which they were harvested for lutein extraction and high-performance liquid chromatography - photodiode array detection (HPLC-PDA) analysis.

Glucose utilization. A time course study was conducted to identify the lag, log, and stationary phases of cell growth, as well as to plot the time course of glucose utilization. The glucose concentration nadir and cell mass peak are used to determine timing of subcultures for serial labeling growth cycles, in order to maximize efficiency of incorporation of ¹³C from glucose into lutein. Four replicate cultures grown in 125 mL flasks under $80 \mu\text{mol} \bullet \text{m}^{-2} \bullet \text{s}^{-1}$ of light were harvested at two-day intervals from day 0 to day 10 and daily from day 11 to day 16. Samples were analyzed for cell mass, glucose content of the media via HPLC-refractive index detection (HPLC-RI), and lutein yield via HPLC-PDA.

Serial culturing and isotopic labeling. For ¹³C isotopic labeling, a serial subculturing method was employed with ¹³C glucose as the sole carbon source in the medium. Initially, three stock culture flasks of the selected lutein-producing cell line were pooled in order to reduce inoculum heterogeneity in the first ¹³C loading cycle culture. After a 14-day culture period, a second ¹³C loading cycle of eight flasks was inoculated with cultures from the first cycle flask. After another 14-day of cycle two growth, a third cycle of 64 flasks was inoculated using cells from the second ¹³C loading cycle. After 14 days of growth, 9 culture flasks from the third cycle were used to inoculate a fourth labeling cycle of 75 cultures. Remaining third cycle culture flasks ($64 - 9 = 55$ flasks) and all culture flasks from the fourth cycle (75 flasks) were harvested after 18 days of growth. Any remaining cells from the first and second cycles not used for inoculation of the second and third cycles, respectively, were not used for further labeling or lutein harvest.

Extraction and purification

Lutein was extracted from hepatic tissue as previously described.³⁸ For analytical screening of carrot cell cultures, lutein was extracted using a method previously developed for carotenoid extraction from tomato cells.³⁹ For preparatory carrot cell extractions, this method was adapted as follows: 30 g of wet, packed cells were homogenized in 50 mL of 0.1% butylated hydroxytoluene in ethanol for 6 min and transferred to a 250 mL bottle. Samples were saponified by the addition of 8.5 mL saturated aqueous KOH (100 g/100 mL) and incubation in a 60°C water bath for 30 min, with vortexing every 10 min. To this, 20 mL of saturated aqueous NaCl (36 g/100 mL) is added, followed by 75 mL hexane and mixing by vortex for 1 min. After allowing phases to separate, the hexane supernatant is removed and set aside; this is repeated twice more, and the combined hexane pool is reduced in a centrifugal vacuum concentrator (Savant, Thermo Scientific, Waltham, MA USA), followed by evaporation to dryness under argon.

The extracts were stored at -20°C until purification by preparatory HPLC.

An HPLC system consisting of an Alliance e2695 separations module and 2998 photodiode array (PDA) detector (Waters, Milford, MA, USA), with column cooling set to 18°C and sample cooling at 4°C was used for all analysis and purification. The HPLC conditions for lutein and alpha-tocopherol analysis were modified from a previously published method.⁴⁰ Briefly, lutein was separated with an isocratic method utilizing a mobile phase of acetonitrile:dioxane:200 mM ammonium acetate in 50%/50% (v/v) methanol/2-propanol:triethylamine (80:15:5:0.1) on a Waters Spherisorb[®] ODS2 150 mm \times 4.6 mm \times 3 μm C18 analytical column. For preparatory HPLC purification, the system was fitted with a 250 mm \times 10 mm \times 5 μm C30 preparatory column (YMC America, Allentown, PA, USA) and C30 guard column. The preparatory separation utilized the same mobile phase as the analytical method, but with omission of ammonium acetate. Flow rate was 4.73 mL/min. Samples were reconstituted in 27 μL of a 100:35 mixture of ethyl acetate:mobile phase, separated by preparatory HPLC, and eluted lutein peaks were collected. Lutein fractions from liver and carrot cell extracts were collected for mass spectrometry analysis (analytical HPLC) or lutein harvest (preparatory HPLC) by collecting PDA detector eluate before lutein peak takeoff and after baseline touchdown. Fractions were dried under argon gas. Fractions from preparatory HPLC for lutein harvest were dissolved in diethyl ether and pooled, ether was removed in a rotary evaporator (Buchi, New Castle, DE, USA) and with argon gas, and a single vial of purified, concentrated ^{13}C -lutein was obtained. This was sampled and analyzed for total lutein content and purity via analytical HPLC-PDA.

In vivo dosing

The Institutional Animal Care and Use Committee of the Oregon National Primate Research Center (ONPRC), Oregon Health and Science University, approved all procedures. A female, 19-year-old rhesus macaque (*Macaca mulatta*) was fed a standard laboratory diet (Monkey Diet Jumbo, LabDiet, St. Louis, MO, USA), supplemented daily for the previous six months with 1 $\mu\text{mol}/\text{kg}/\text{day}$ (570 $\mu\text{g}/\text{kg}/\text{day}$) of unlabeled lutein (FloraGlo[®] beadlets, 5% lutein, DSM, Herleen, the Netherlands). The animal was anesthetized with ketamine (10 mg/kg intramuscular injection), intubated with an orogastric catheter (Rusch, Teleflex, Morrisville, NC, USA), and dosed with ^{13}C -labeled lutein solubilized in mixed monoglyceride/diglyceride (MDG) oil (Abbott Nutrition, Columbus, OH, USA). Purified lutein was dissolved in 4 mL of chloroform and thoroughly mixed into 2.09 mL of MDG oil. The chloroform was removed from the lutein-oil mixture via evaporation under argon and the resulting mixture was sampled in duplicate for determination of final lutein concentration. In order to prepare the orogastric catheter (size 28 Fr) for gastric lutein dosing, using a 10 mL polypropylene syringe, unlabeled MDG oil was drawn through the catheter and into the syringe, followed by full expulsion back into the

oil reservoir; this was performed for eight cycles to ensure full laminar coating of the catheter walls, thus minimizing lutein loss on the interior of the catheter. Next, 4 mL of unlabeled MDG oil was drawn into the catheter, followed by the lutein-MDG oil dose, and finally followed by an additional 2 mL of unlabeled MDG oil. After dose administration to the monkey, approximately 3 mL nonfat milk was placed into the vial previously containing the lutein-oil dose mixture, and the tube was agitated vigorously. Additional nonfat milk was added to this mixture to bring the total volume to 15 mL, it was drawn into the syringe, and this volume was used to chase the lutein dose and rinse remaining contents of the orogastric catheter into the monkey's stomach. All equipment used to prepare and administer the lutein dose (e.g., pipette tips, Pasteur pipettes, syringe, orogastric catheter, etc.) was retained and washed thoroughly with diethyl ether to recover remnant lutein. This diethyl ether wash was extracted, saponified, and analyzed via HPLC to determine dosing efficiency.

Three days after oral ^{13}C -lutein dosing, the animal was euthanized under deep sodium pentobarbital anesthesia. Tissues were collected and snap-frozen in liquid nitrogen, and samples were shipped on dry ice from the ONPRC to the University of Illinois at Urbana-Champaign for analysis.

Two rhesus macaques (male, 161 days old; female, 220 days old) were fed unlabeled lutein-supplemented diets for four months, but were not dosed with ^{13}C -lutein. Tissues were collected in the same manner as for the ^{13}C lutein-dosed animal and liver tissue was used as negative control for detection of ^{13}C lutein in liver.

Mass spectrometry

Multiple analytical approaches were used in the study of ^{13}C -lutein in carrot and monkey liver extracts. For initial carrot culture extract measurements, laser desorption ionization-mass spectrometry (LDI-MS) in reflectron positive polarity mode was used. In these experiments, dried carrot extracts were reconstituted in 10 μL of 200-proof ethanol. The resulting solutions had visible yellow color. 0.3 μL of the solution was spotted on a metal sample plate and analyzed with a UltrafleXtreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Billerica, MA, USA) equipped with solid-state UV Smartbeam II laser. The laser operated at 1 kHz repetition firing. The mass spectra each averaged 1000 laser shots. Mass resolving power and mass accuracy was 27,000 and 30 ppm, respectively.

For the analyses of lutein in HPLC-purified monkey liver and carrot culture fractions, ultrahigh performance liquid chromatography-triple quadrupole-electrospray ionization-mass spectrometry (UHPLC-TQ-ESI-MS) was used to evaluate ^{13}C -lutein content. An EVOQ Elite[™] TQ-ESI-mass spectrometer (Bruker Daltonics), equipped with VIP heated electrospray and APCI ion source, was tuned using polytyrosine calibration and tuning mixture (Bruker Daltonics, cat no. 394127601). The instrument operated in polarity switching mode with the following settings: (+) spray current 15.0 μA ; (–) spray current 15.0 μA ; cone temperature 250°C ; cone gas flow 10 arbitrary units (AU); heated probe temperature 450°C ; probe gas flow 15 AU; nebulizing gas

flow 60 AU; collision gas pressure 1.5 mTorr. 0.7 unit resolution was set for both Q1 and Q3 quadrupoles.

Dried monkey liver fractions were reconstituted in 25 μ L of ethanol and 25 μ L of acetonitrile was added to the sample. Ten microliters of this solution was injected onto a C18 column (50 mm, 1.7 μ m particle size, Fortis Technologies Ltd, UK) using a CTC Autosampler with mitigated carryover. C18 columns have demonstrated good performance in separation of lutein (reviewed by Rivera and Canela-Garayoa⁴¹). Analytes were separated using an isocratic UHPLC method consisting of 100% acetonitrile running at 300 μ L/min for 8 min.

Deuterated GABA was spiked into all samples as an internal standard to monitor system performance. Determination of instrument response linearity, as well as quantification of analytes, was accomplished with calibration curves of external analyte standards. Multiple reactions monitoring (MRM) provided information on analyte identity. Three different transitions for each analyte parent ion were monitored during the entire MRM run. Previously reported ¹²C-lutein MRM transitions were monitored (reviewed by Rivera and Christou⁴²), as well as those suggested by METLIN (metlin.scripps.edu) and MASSBANK (<http://www.massbank.jp/>) metabolomics databases. The following transitions found during product scan analysis of ¹³C-lutein-related ions, generated from LC fractions of carrot culture extracts, were also used in subsequent experiments: [M + H]⁺ ¹³C-lutein m/z 609.6 \rightarrow 105.9, 609.6 \rightarrow 243.5, 609.6 \rightarrow 430.0; [M + H-H₂O]⁺ ¹³C-lutein m/z 591.6 \rightarrow 143.6, 591.6 \rightarrow 503.2, 591.6 \rightarrow 487.0; [M]⁺ ¹³C-lutein m/z 608.6 \rightarrow 176.7, 608.6 \rightarrow 389.1, 608.6 \rightarrow 123.3. Data acquisition and processing was managed by Bruker MS Workstation software (Version 8.1.2, Bruker Daltonics). Limits of detection are in range of high parts per trillion (ppt) to low parts per billion (ppb).

Statistical analysis

Two-way analysis of variance (ANOVA) was used for all analyses of lutein production in cell culture, with the exception of analysis of lutein and alpha-tocopherol concentrations over four serial growth cycles, in which case repeated measures ANOVA was used. *Post hoc* Tukey's Studentized range test was used to identify group differences. All analysis was done in SAS version 9.3 (SAS Institute, Cary, NC, USA). Mass spectrometry data were summarized and analyzed using Excel to determine average and standard deviation. Correlation coefficients were calculated using the correlation function incorporated into Excel's Analysis ToolPak.

Results and discussion

Callus and solution culture screening

From the four tissue types sampled (true leaf, root, hypocotyl, cotyledon) from the seven carrot varieties studied (Atomic Red, Amarillo, Jaune Obtuse du Doubs, Yellow Solaris, Yellowstone, Purple Dragon, Purple Sun), 66 total callus cell lines were generated. These were grouped by the combination of varietal and tissue source, resulting in 17

unique callus tissue types (Table 1S, Supplementary material). Replication – the number of cell lines generated from a callus tissue type within a given varietal – ranged from 1 to 6. Callus tissues were sampled for lutein content to determine promising cell lines from which to establish solution cultures. As can be seen in Table 1S (Supplementary material), high variability was evident across the 17 different tissues sampled (%CV = 59%). There were no overall patterns in callus tissue lutein accumulation, as tissue source did not predict lutein yield across carrot varieties (e.g. root tissues ranged from 0.00 μ g/g in Atomic Red to 3.59 μ g/g in Yellowstone) and tissue sources from the same varietal at times differed by a considerable amount (e.g. 6.83 μ g/g in Jaune Obtuse hypocotyl tissue vs. 1.91 μ g/g in root tissue). However, trends emerged in carrot varieties, as Atomic Red was a very poor lutein producer in both callus tissues generated, while, in general, the yellow varieties (Amarillo, Yellow Solaris, and Yellowstone) tended to have higher yields; this was especially true in lines derived from hypocotyl tissues. Notably, the yellow carrot varietal Jaune Obtuse du Doubs showed highly dimorphic lutein tissue accumulation, as the hypocotyl-derived callus had the highest concentrations of all tissues tested (6.83 μ g/g), while root tissue was below the average for all tissues (1.91 μ g/g; mean = 2.62 μ g/g). The overall difference between yellow and red carrot varieties agrees with genetic studies of carotenoid biosynthetic genes during carrot development, as yellow carrots, such as the Yellowstone varietal, demonstrate upregulation of lycopene *e*-cyclase (*Lcye*), which drives carotenoid accumulation towards lutein, while red varieties demonstrate increased expression of zeta-carotene desaturases 1 and 2 (*Zds1*, *Zds2*) and relatively low expression of *Lcye*, promoting lycopene accumulation.⁴³ The anthocyanin-rich purple varieties Purple Dragon and Purple Sun exhibited moderate lutein production in all tissues tested.

Five callus tissue types were selected for initiation into solution culture – Amarillo root, Jaune Obtuse du Doubs hypocotyl, Yellow Solaris root, and hypocotyl and root from the Yellowstone varietal (Table 1S, Supplementary material). Selection of callus tissues to initiate into solution culture was based on several factors, most obvious among them being lutein accumulation. In that regard, Jaune Obtuse hypocotyl, Yellowstone hypocotyl, and Yellowstone root tissues ranked in the top four of all callus tissues in lutein accumulation (6.83 μ g/g, 3.63 μ g/g, and 3.59 μ g/g, respectively), and on this basis, were selected for initiation into solution culture. While the mean level of lutein accumulation in the Yellow Solaris root callus tissue was only near the median in lutein content (2.55 μ g/g, ranked 7/17), two of the four replicates within that callus tissue type had levels of lutein that were not quantifiable, while the other two replicates accumulated greater than 5.0 μ g/g lutein. Therefore, those two lutein-accumulating Yellow Solaris root callus lines were initiated into solution culture on the basis of their individual lutein production, despite the group heterogeneity. Finally, while the Amarillo root callus tissue was a poor lutein producer (0.96 μ g/g, ranked 15/17), it accumulated α -tocopherol more than any other callus tissue that had not already

been selected for its lutein production (Jaune Obtuse hypocotyl and Yellowstone root also accumulated high levels of α -tocopherol). Production of a uniformly labeled α -tocopherol tracer was of separate interest to our research team and so the Amarillo root callus tissue was the final tissue selected for initiation into solution culture.

Solution culture optimization

Solution cultures generated from tissue callus underwent two screening cycles before cell culture optimization, to verify that lutein production in solution culture stably reflected solid callus levels. While Yellowstone hypocotyl and Yellowstone root callus tissues were the second- and third-highest lutein-accumulating callus tissues, they did not maintain their level of production (on a $\mu\text{g/g}$ basis) in solution culture (Table 1S, Supplementary material). Yellowstone hypocotyl decreased by 31%, from 3.63 $\mu\text{g/g}$ to 2.50 $\mu\text{g/g}$, while the Yellowstone root line decreased even further, by 74% (3.59 $\mu\text{g/g}$ to 0.93 $\mu\text{g/g}$). The two highest lutein producers in solution culture, on the basis of $\mu\text{g/g}$ accumulation, were Jaune Obtuse hypocotyl tissue and, surprisingly, Amarillo root tissue. Amarillo root lutein production dramatically increased when transferred from callus to solution culture (0.96 $\mu\text{g/g}$ callus to 4.14 $\mu\text{g/g}$ in solution, 431% increase) and, although Jaune Obtuse hypocotyl did decrease slightly when transferred to solution culture, (6.83 $\mu\text{g/g}$ callus to 5.17 $\mu\text{g/g}$ in solution, 24% decrease), it was still the highest lutein producer of the solution cultures tested. Yellow Solaris root experienced a slight increase in lutein production during the transition (2.55 $\mu\text{g/g}$ callus to 2.85 $\mu\text{g/g}$ in solution, 12% increase). Therefore, Jaune Obtuse hypocotyl and Amarillo root solution cultures were used for further optimization of lutein production. These data demonstrate the heterogeneous metabolic adaptation of plant cells to novel culture conditions and underscore the need to screen many lines at once in order to increase the probability of selecting an ideal high-yield biofactory.

Culture media and carbon source

Standard plant cell growth media formulations utilize sucrose as the carbon source.^{44,45} However, the only commercially available, economically priced, and uniformly labeled ([U]) ^{13}C carbohydrate is glucose. Therefore, determination of suitability of glucose as a sole carbon source for lutein production needed to be determined in our system. Additionally, various combinations of vitamins, minerals, and nitrogenous salts may be used for plant solution culture⁴⁶ and may affect cell growth and lutein yield. To these ends, a $2 \times 2 \times 2$ factorial experiment was conducted to determine the production of lutein in solution culture *in vitro* in the Amarillo root and Jaune Obtuse hypocotyl cell lines, providing either glucose or sucrose as the sole carbon source, in either B5^{44,45} or M&S⁴⁷ culture media. As seen in Figure 2, while there were no statistically significant three-way interactions, we did observe a two-way interaction between cell line and culture media. While B5 media supported lutein production in either the Amarillo or Jaune Obtuse cell lines, Amarillo cultures in M&S media

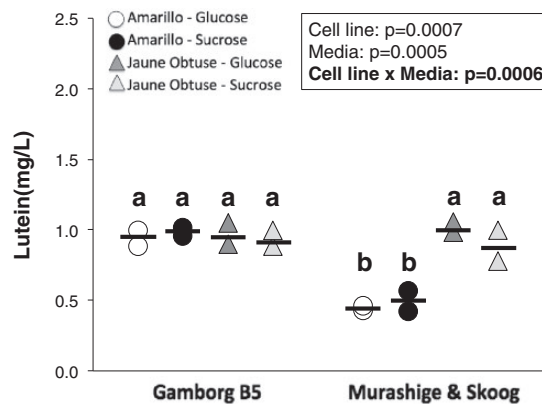


Figure 2 Lutein yield (mg/L solution) in Amarillo or Jaune Obtuse du Doubs carrot cell lines cultured with glucose or sucrose as the sole carbon source and in B5 or M&S plant cell culture media. Data points are duplicates for each treatment combination. Significant interactions and main effects ($p < 0.05$) calculated by two-way ANOVA are noted. Different letters above groups indicate statistical significance (Tukey's adjusted $p < 0.05$)

experienced a significant decrease in production of approximately 51%. Previous work has shown that carrot cells grown in liquid culture preferentially take up *glucose* from the culture medium and do not assimilate media sucrose directly into cellular metabolites.⁴⁸ Despite this preference for glucose, cell growth was not inhibited by sucrose as a sole supply of carbon in the media, likely due to hydrolysis of extracellular sucrose to its constituent fructose and glucose monomers by secreted invertase enzyme, allowing the uptake of glucose for growth and metabolism.⁴⁸ In agreement with this previous work, we did not observe an impact of carbon source (glucose vs. sucrose) on lutein production.

Light/dark preference and light intensity

Lutein, like other carotenoids in photosynthetic plants, serves not only as a photoprotective antioxidant, but also it plays a key role in photosynthetic light-harvesting.^{49,50} In fact, lutein is the predominant carotenoid present in photosynthetic leaves, accounting for 40% of total carotenoids.⁵¹ Thus, we tested whether light presence or intensity affected lutein production in selected carrot cell cultures *in vitro*. Glucose and B5 media were used for culture, per results from the previous experiment. Light treatment ($40 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), compared to light exclusion by covering flasks in aluminum foil, resulted in greater lutein accumulation in several carrot cell lines tested (data not shown). Next, we tested whether a higher-intensity light treatment would further boost lutein yields in the Amarillo and Jaune Obtuse carrot cell lines. Lutein yields in the Amarillo line, but not the Jaune Obtuse line, doubled ($p < 0.05$) when exposed to an $80 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ light treatment compared to $40 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ (Figure 3). In agreement with our findings, previous work in kale⁵² has also shown that increased light irradiance results in increased lutein content.

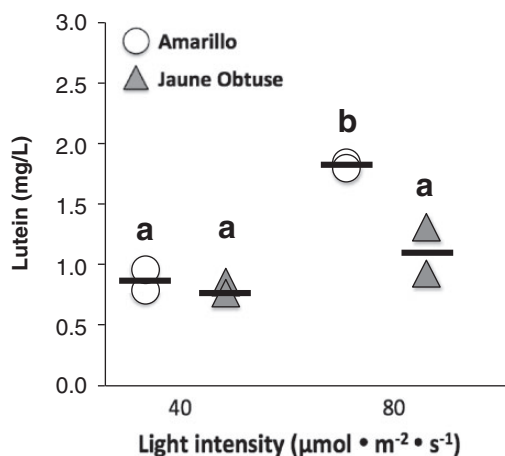


Figure 3 Lutein yield (mg/L solution) in Amarillo or Jaune Obtuse du Doubs carrot cell lines, cultured in 125 mL flasks under $40 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ or $80 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ light treatment. Data points are duplicates for each treatment combination. Different letters above groups indicate that statistical significance calculated by *post hoc* means separation following two-way ANOVA (Tukey's adjusted $p < 0.05$)

Flask size

Culture flask size impacts culture media surface area, evaporation, gas exchange, and oxygenation; these factors may impact cell growth and metabolite production.^{53,54} Therefore, we tested the effects on lutein production of doubling flask capacity from 125 mL (containing 40 mL media) to 250 mL (containing 80 mL media). Doubling flask size (and concomitantly doubling media volume) did not significantly affect lutein yield in either the Amarillo or Jaune Obtuse cell lines (data not shown). However, equivalent lutein production in 125 mL flasks compared to 250 mL flasks is preferable, as it limits microbial contamination risk; a contaminated 125 mL flask represents a smaller fraction of the overall cell mass being cultured.

After testing of the above variables, an Amarillo root tissue cell line grown in 125 mL flasks in B5 media under $80 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ light with glucose as the sole carbon source achieved peak lutein production; thus, these were the final conditions chosen for further work.

Glucose utilization timecourse

In order to identify the optimal harvest day to allow maximal ¹³C uptake by carrot cultures, a glucose utilization timecourse study was performed. Total cell mass was weighed and glucose concentrations in the media were assayed via HPLC-RI at 11 timepoints over 16 days. Media glucose was depleted by 14 days, while cell mass reached a maximum at 14 days (Figure 4). Cell mass remained at maximum at day 16, despite a depletion of glucose in the media at 16 days.

In vitro isotopomeric labeling

Lutein yield decreased slightly during ¹³C labeling. While yields in cycles 1 and 2 were equivalent ($3.83 \pm 0.02 \mu\text{g/g}$ and $3.73 \pm 0.07 \mu\text{g/g}$, respectively), lutein production dropped by 15% in cycle 3 compared to cycle 1 (to

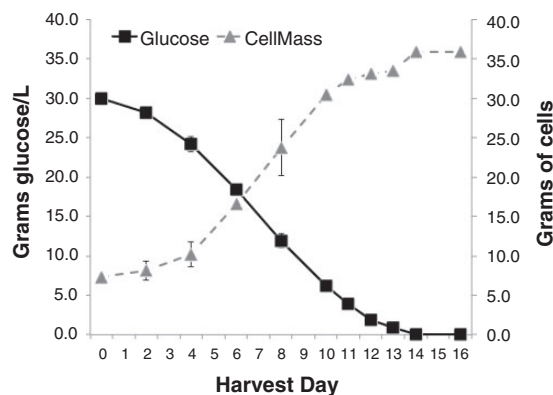


Figure 4 Cell mass (in grams) and glucose concentration (in g/L solution) in Amarillo cell line cultures over 16 days. Error bars (some not visible) represent SEM of 3–4 total replicates for each data point over the course of two independent experiments

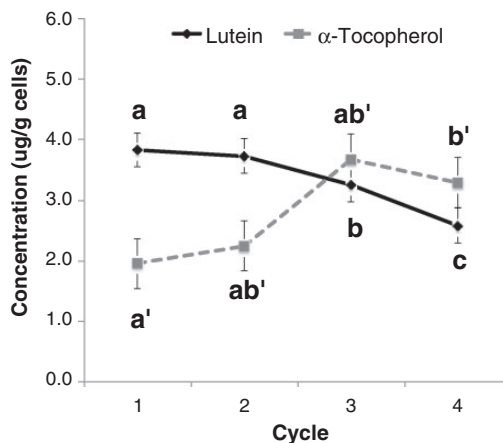


Figure 5 Lutein and alpha-tocopherol concentrations ($\mu\text{g/g}$ cells) in Amarillo carrot cells, cultured under optimized conditions, across two ¹³C loading cycles (cycles 1 & 2) and two ¹³C loading/harvest cycles (cycles 3 and 4). Error bars represent SEM of 2–3 replicates for each data point. One-way repeated measures ANOVA with *post hoc* Tukey's test analysis was used to calculate statistical significance. Statistically, lutein and alpha-tocopherol were analyzed separately, as indicated by absence or presence of primes (e.g. a vs. a'). Different letters denote statistically different data points (Tukey's adjusted $p < 0.05$)

$3.26 \pm 0.10 \mu\text{g/g}$) and 33% in cycle 4 compared to cycle 1 (to $2.58 \pm 0.24 \mu\text{g/g}$) (Figure 5), both statistically significant deviations. This is consistent with previous work in our laboratory on tomato cells, where lycopene and total carotenoid production decreased with progressive ¹³C culture enrichment.³² In the same study, time to media glucose depletion shifted three days – from day 9 to day 12 of culture – between the ¹³C loading phase (cycle 1) and ¹³C labeling/harvest phase (cycles 2 and 3),³² potentially attributable to a longer lag in cell growth with further enrichment of cellular ¹³C pools. Due to the precious nature of the labeled cells in the present study, cell mass was not assessed in the ¹³C-lutein labeling experiment, preventing definitive conclusions about the cause of the observed decreased lutein yield. However, it is known that C₃ plants, such as tomatoes and carrots, discriminate against ¹³C in carbon

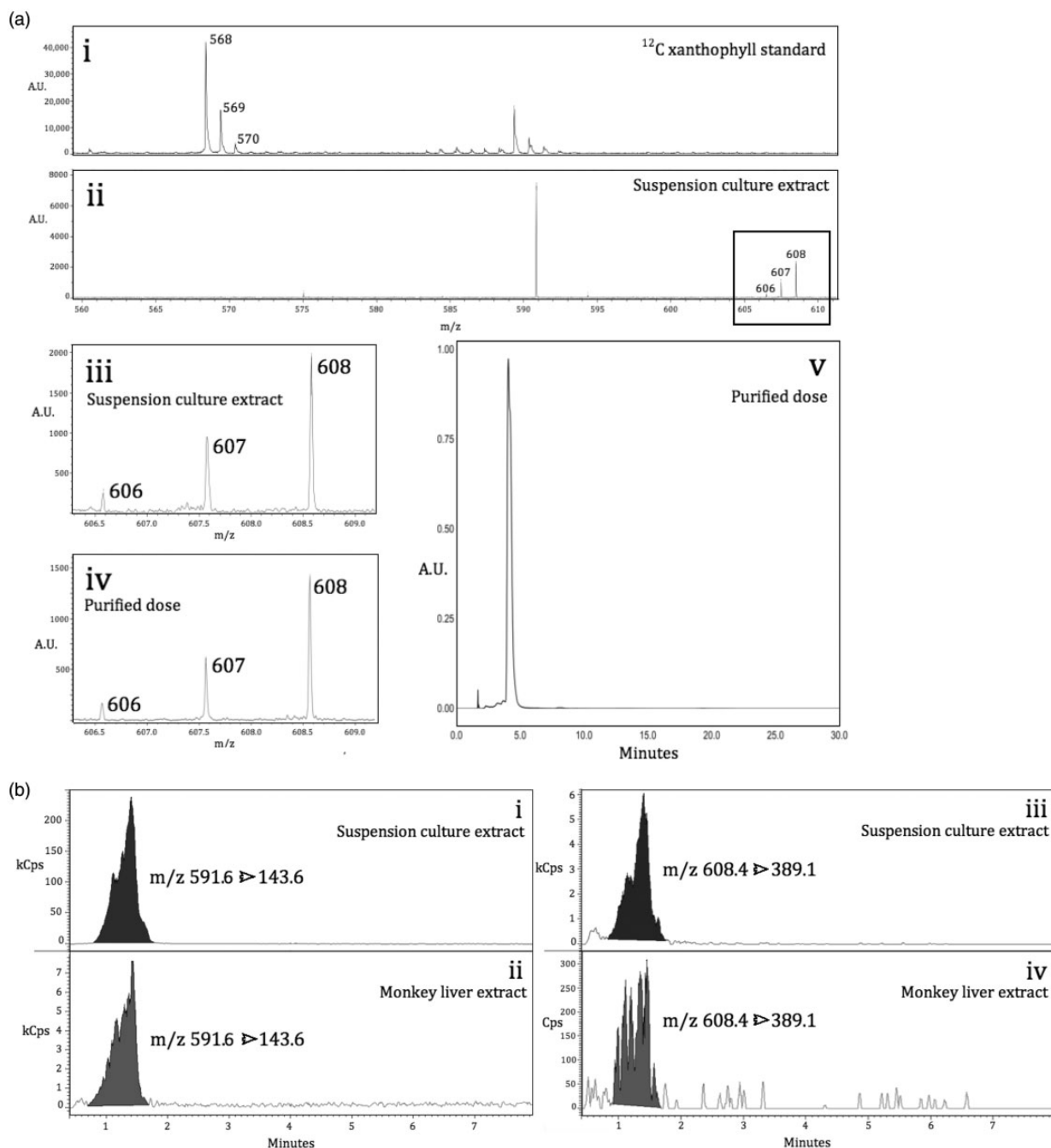


Figure 6 ^{13}C -lutein is successfully biosynthesized and purified from carrot cell solution culture, and can be detected in monkey liver four days after oral gavage. (A) Representative mass spectra and chromatogram of carrot extracts and purified lutein dose. (i) LDI-MS mass spectrum of unlabeled xanthophyll standard; (ii) LDI-MS mass spectrum of extract from ^{13}C lutein harvest cycle 3 (m/z range of ^{13}C -lutein is indicated by box outline); (iii) magnification of ^{13}C -lutein m/z range from (ii); (iv) ^{13}C -lutein m/z range from LDI-MS mass spectrum of purified lutein dose [no isotopomers of m/z lower than 606 were detected]; (v) HPLC-PDA chromatogram demonstrating purity of prepared ^{13}C -lutein dose, sampled immediately prior to oral gavage in a rhesus macaque monkey. (B) Representative UHPLC-TQ-ESI-MS mass chromatograms of MRM transitions characteristic of ^{13}C -lutein ions. (i) $[\text{M} + \text{H} - \text{H}_2\text{O}]^+$ ^{13}C -lutein ions were detected in carrot cell extracts and (ii) the liver of a monkey dosed with ^{13}C -lutein. (iii) $[\text{M}]^+$ ^{13}C -lutein ions were detected in carrot cell extracts and (iv) the liver of a monkey dosed with ^{13}C -lutein

fixation (in favor of ^{12}C), impacting photosynthetic efficiency.³⁷ While photosynthesis is bypassed by provision of glucose in the media of our cultures, enrichment of ^{13}C may impact still other metabolic processes to retard cell mass accumulation, synthesis of secondary metabolic products, or both. It is interesting to note, however, that α -tocopherol levels in the present study significantly increased in

cycle 4, compared to cycle 1 (Figure 5), suggesting that the effect of ^{13}C loading on metabolite synthesis may be pathway-specific.

Samples of solution cultures were taken during cycles 1 and 2 (^{13}C loading cycles) and cycles 3 and 4 (^{13}C labeling/harvest cycles) for measurement of ^{13}C lutein isotopomer profile by LDI-MS. Representative mass spectra acquired

Table 1 Isotopomer profiles in carrot cell extracts and purified ¹³C-lutein dose.

		¹³ C ₄₀ H ₅₆ O ₂	¹² C ¹³ C ₃₉ H ₅₆ O ₂	¹² C ₂ ¹³ C ₃₈ H ₅₆ O ₂	¹² C ₃ ¹³ C ₃₇ H ₅₆ O ₂
<i>In vitro</i> ^a	Cycle 1*	55.6%	28.0%	16.4%	n.d.
	Cycle 2	63.8% ± 1.4%	28.2% ± 1.2%	8.0% ± 0.2%	n.d.
	Cycle 3	66.8% ± 1.8%	26.6% ± 1.9%	6.6% ± 0.6%	n.d.
	Cycle 4*	64.9%	25.5%	9.6%	n.d.
Purified dose^b		64.7 ± 0.9%	27.0 ± 0.4%	8.3 ± 0.9%	n.d.

Isotopomer profiles in each of the carrot cell culture ¹³C loading cycles (1 and 2) and ¹³C harvest cycles (3 and 4), as well as in the purified lutein dose prepared for treatment of the animal. ^aFor the *in vitro* carrot samples, carrot cells were extracted, lutein purified by HPLC, collected, and samples were dried and analyzed by LDI-MS. Data represent means ± SEM of four measurements (two technical replicates for each of two LC fractions), except for Cycles 1 and 4 (marked with an asterisk [*]); in these cases, there was not enough material for replication and samples were analyzed once. ^bFor the purified lutein dose, lutein was extracted, dried, and analyzed by LDI-MS. Data represent mean ± SEM of four technical replicates. Unlabeled lutein and lutein labeled with ¹³C at 37 or fewer possible carbons was not detected in any sample (n.d., not detected).

from cycle 3 extracts are shown in Figure 6(Aii) and (Aiii). For comparison, a mass spectrum of an unlabeled, lutein standard (xanthophyll from marigold, X6250, Sigma) is shown in Figure 6(Ai); unlabeled lutein has a monoisotopic molecular mass of 568.42, while lutein fully labeled at all 40 carbons has a monoisotopic molecular mass of 608.56 (calculated using www.chemcalc.org⁵⁵). As anticipated, ¹³C enrichment increased with culture cycles, from observed monoisotopic patterns of 55.6% uniformly labeled (40/40 possible carbons) in cycle 1 to 64.9% uniformly labeled in cycle 4 (Table 1). An inverse pattern was observed in the percent of isotopomers labeled at 38/40 carbons, which decreased from 16.4% in cycle 1 to 9.6% in cycle 4. Additionally, the data suggest an enhancement in isotopic enrichment of lutein between the loading phase (cycles 1 and 2) and the harvest phase (cycles 3 and 4). Not only does this support our use of lutein harvested from both cycles 3 and 4 in subsequent dosing, but it also lends credence to the hypothesis that a metabolic threshold of ¹³C cellular loading exists between the loading and harvest phases, and that the co-occurrence with decreased lutein accumulation at this phase interface may be a sign of altered cellular metabolism.

In vivo dosing

Carrot cells from cycles 3 and 4 were extracted, and ¹³C-labeled lutein was purified with reverse-phase HPLC, pooled, and sampled for analysis prior to *in vivo* dosing in rhesus macaque. The lutein dose was sampled both before and after shipment on dry ice to the Oregon National Primate Research Center for dosing. Lutein in the dose was not affected during shipment, as lutein purity was 96.0 ± 0.02% and 96.0 ± 0.60% before and after shipment, respectively. A representative chromatograph of the lutein dose is shown in Figure 6(Aiv). The isotopomeric profile of the dose reflected a high degree of ¹³C labeling, with 64.7 ± 0.9% uniformly labeled (40/40 carbons), 27.0 ± 0.4% labeled at 39/40 carbons, and 8.3 ± 0.9% labeled at 38/40 carbons (Table 1). A representative mass spectrum of the lutein dose is shown in Figure 6(Av). As in the labeled carrot cell extracts, no lutein with ¹³C labeling at 37 or fewer of the 40 possible positions was detected in the purified lutein dose (Table 1).

HPLC-PDA analysis of samples of the lutein dose taken prior to administration indicated that dose to be delivered was 1.98 mg of total ¹³C-labeled lutein. All equipment used to handle lutein during the dose preparation and delivery protocol (e.g., pipette tips, centrifuge tubes, Pasteur pipettes, Rusch catheter, syringe, etc.) were saved and stored at -80°C until analysis for remnant lutein. HPLC-PDA analysis of remnant lutein revealed that only 57 µg of 1.98 mg lutein was *not* delivered to the animal, for an effective dosing efficiency of 97.2%; thus, it was calculated that 1.92 mg of ¹³C-labeled lutein was delivered into the stomach of the animal subject.

Hepatic ¹³C-lutein detection

Our next goal was to measure hepatic ¹³C-lutein in the dosed animal using several MS-based approaches to demonstrate, in a qualitative manner, that the ¹³C-lutein was absorbed from the gut and accumulated to detectable levels in the liver. A number of molecular ions characteristic for ¹³C-lutein were detected in extracts of carrot cells (Figure 6(Bi, iii)) and monkey liver (Figure 6(Bii, iv)) using an MRM approach with UHPLC-TQ-ESI-MS. UHPLC-TQ-ESI-MS analysis of two LC fractions of hepatic extract of the ¹³C-lutein-fed monkey confirmed the presence of unlabeled (Table 2S, Supplementary material) and ¹³C-labeled (Table 3S, Supplementary material) lutein. This conclusion is based on the detection of multiple characteristic parent and fragment ions. Additionally, relative areas of the MRM signals acquired from the unlabeled lutein standard (xanthophyll) were similar to signals indicative of unlabeled lutein detected in livers of three animals with correlation coefficients ranging between 0.89 and 0.99 (Tables 2S and 4S, Supplementary material). Similar comparison of MRM ¹³C-lutein-related signals in carrot extract fraction and extract of liver of the monkey fed with ¹³C-lutein produced a correlation coefficient of 0.85 (Table 3S, Supplementary material). The results corroborate the HPLC-PDA measurements of the fractions. Additionally, UHPLC-TQ-ESI-MS measurements of two LC fractions of extracts from livers of two reference monkeys fed unlabeled lutein (i.e. not dosed with ¹³C-lutein) unambiguously identified unlabeled lutein (Table 4S, Supplementary material) but not ¹³C-labeled lutein (Table 5S, Supplementary material).

Unlike with the MRM approach, characteristic ^{13}C -lutein signals were not observed in hepatic extracts using LDI-MS, likely due to relatively low abundance of the related ions and chemical complexity of the samples.

In summary, we have demonstrated a method for the efficient production, extraction, and purification of highly enriched ^{13}C -lutein from carrot (*Daucus carota*) solution culture *in vitro*, which represents a novel biofactory for lutein tracer production. Additionally, subsequent to oral dosing of an adult rhesus monkey (*Macaca mulatta*) with ~ 1.9 mg ^{13}C -lutein, we were able to detect ^{13}C -lutein in hepatic tissue using UHPLC-TQ-ESI-MS via MRM. Future work will characterize the deposition and metabolism of ^{13}C -lutein metabolites in primate tissues.

Authors' contribution: All authors participated in the design, interpretation of the studies, analysis of the data, and review of the manuscript. JWS, RBR, SJ, SSR, LW, and MN conducted the experiments; JWS, RBR, SJ, SSR, LW, JVS, MN, MJK, and JWE wrote and edited the manuscript. JWS current affiliation: Department of Environmental Health and Engineering, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, MD 21205, USA.

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DECLARATION OF CONFLICTING INTERESTS

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