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Review

Intrinsic stable isotope labeling of plants for nutritional investigations in humans

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Although plant foods provide an array of nutrients in the human diet, our knowledge of how efficiently these nutrients are absorbed has been limited by our ability to selectively monitor their absorption from a complex food matrix. Intrinsic labeling of plants with low-abundance stable isotopes can provide a safe, traceable product to investigate absorptive phenomena in the gut. Various techniques, including hydroponic culture, stem injection, and atmospheric labeling, have been used to introduce isotopes of either minerals or carbon to the plant. New, alternative possibilities exist for labeling plants with deuterium through the use of heavy water, or for using isolated root cultures. Each labeling situation offers some unique concern, related to the plant, the nutrient, or the isotope expense, that must be addressed to generate a useful and affordable product. Attention to the mechanisms and pathways by which a specific nutrient is transported throughout the plant will aid in the selection of an effective labeling approach that will (1) maximize the recovery of isotope in the edible tissues, and (2) ensure that the isotope is incorporated into endogenous compounds and compartments in the same manner as that of the normal high-abundance isotopes. Intrinsically labeled plants may be used in an unlimited number of human bioavailability investigations focusing on essential nutrients, secondary plant metabolites, or unique phytochemicals. (J. Nutr. Biochem. 8:164–171, 1997) © *Elsevier Science Inc. 1997*

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Introduction

Plant foods are an important source of essential nutrients in the human diet. Fruits, vegetables, and seed crops can provide an array of minerals, amino acids, carbohydrates, lipids, nucleic acids, vitamins, and provitamins. In addition, various phytochemicals that may have health benefits, such as for cancer prevention, also are available from plant sources.[1,2] Our knowledge of how efficiently these dietary constituents are absorbed in the human gut is limited, primarily because of the problems inherent in selectively monitoring the absorption of a nutrient from a specific food source within a complex diet, and in discerning newly absorbed nutrients or phytochemicals from previously existing levels in the body. This lack of knowledge is unfortunate, because our ability to provide reliable nutritional dietary guidelines is dependent on accurate assessments of nutrient bioavailability from commonly consumed foods.[3]

Intrinsic labeling of plants with stable isotopes provides a safe and traceable tool for quantifying nutrient absorption in humans from specific foods.[4] Stable isotopes have been used in human investigations for a number of years,[5] and are an acceptable and preferred alternative to radioisotopes. Most nutritionally important elements have one or more stable isotopic forms, and generally at least one form that

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Table 1 Stable isotopes of selected elements important to human nutrition (adapted from Ref. 6)

Element	Isotope	Natural abundance (%)
Hydrogen	^{1}H	99.985
	^{2}H	0.015
Carbon	^{12}C	98.90
	^{13}C	1.10
Nitrogen	^{14}N	99.63
	^{15}N	0.37
Magnesium	^{24}Mg	78.99
	^{25}Mg	10.00
	^{26}Mg	11.01
Calcium	^{40}Ca	96.941
	^{42}Ca	0.647
	^{43}Ca	0.135
	^{44}Ca	2.086
	^{46}Ca	0.004
	^{48}Ca	0.187
Iron	^{54}Fe	5.90
	^{56}Fe	91.72
	^{57}Fe	2.10
	^{58}Fe	0.28
Copper	^{63}Cu	69.17
	^{65}Cu	30.83
Zinc	^{64}Zn	48.6
	^{66}Zn	27.9
	^{67}Zn	4.1
	^{68}Zn	18.8
	^{70}Zn	0.6

occurs in nature at low abundance (*Table 1*). When growing a plant in a medium enriched with a low-abundance stable isotope, the isotope will be incorporated into endogenous compounds and compartments in a manner almost identical to that of the normal high-abundance isotopes (although in some cases, there may be minor isotope discrimination effects). After ingestion of the labeled food, the analysis of isotope in collected breath, blood, urine, and/or fecal samples by mass spectrometry techniques enables the determination of percent nutrient absorption.[7,8]

The primary deterrent to intrinsic labeling of plants has been the high cost of generating the plant material. Stable isotopes are quite expensive, relative to radioisotopes, and often a great deal of isotope loss must be tolerated to generate food that contains an adequate isotopic dose. After a stable isotope is introduced into a plant, it is transported to and used by all of the plant's tissues, with only a fraction being recovered in the edible portion.[4] This problem has led many researchers to study absorption phenomena using extrinsically labeled foods, which involves mixing an exact dose of isotope into a food source. In the case of mineral nutrients, the isotope usually is supplied as an inorganic salt, which may not be the chemical form occurring endogenously within the plant material. Nonetheless, extrinsic labeling sometimes has given mineral bioavailability results comparable to those of intrinsically labeled foods, at least when studied using radioisotopes,[9,10] and has prompted the idea that a common, exchangeable pool is established for certain minerals in the intestinal lumen. For some minerals, however, extrinsic labeling yields a poor estimate of endogenous nutrient absorption for certain foods,[11] indicating that only intrinsic labeling can provide a fully accurate result. Intrinsically labeled food also is ideal for assessing the effects of food preparation or processing on final nutrient bioavailability, and with respect to complex molecules, that may not be commercially available in a stable isotope-labeled form (e.g., specific proteins, vitamins, unique phytochemicals), intrinsic labeling is the only means for generating and providing them in a human investigation.

Various experimental techniques and conceptual approaches are available for labeling plants to generate food products of appropriate isotopic enrichment, and with an acceptable level of isotope recovery. In general, if attention is paid to the mechanism by which plants transport a nutrient to an organ of interest, the application of the isotope can be spatially or temporally directed such that a high recovery of isotope can be achieved in the edible tissues. This review will outline the basic techniques available for labeling plants and will present a number of issues that should be considered when performing these procedures.

Methods for labeling plants

Hydroponic culture

Labeling via hydroponic culture is an appropriate technique for nutrients normally absorbed through the plant's root system. Hydroponics involves growing plants in an aerated nutrient solution, with mineral nutrients provided at concentrations and molar quantities sufficient to maintain optimal growth and development of the plant.[12–15] The tissues harvested from hydroponically grown plants are comparable to those of field-grown material, because the mechanisms of root ion absorption and whole-plant transport and deposition are identical to those of a field-grown plant. For isotopic labeling, hydroponic culture is recommended over soil culture because isotope loss can be quite high in soils, because of adsorption of ions onto soil particles. Hydroponic systems have been used to label plants with a number of stable isotopes, including those of Ca,[16] Mg,[17] Zn,[18] and N.[19]

Two general approaches are available for growing plants hydroponically: static systems and recirculating systems. Static systems involve single growth containers that are best used when small quantities of plants are grown, or when labeling is planned only for a short duration. Recirculating systems involve interconnected growth containers with solution pumped throughout the system[19–21] and are ideal for long-term growth and labeling. They enable the investigator to simultaneously regulate nutrient levels for a large number of plants, and ensure that all the plants in a study are receiving comparable levels of nutrients, including the stable isotope of interest. Absorbed nutrients and water are replenished continuously in recirculating systems, such that solutions are never drained and replaced. Although this requires monitoring of nutrient concentrations, to maintain them between deficient and toxic limits,[22,23] the recirculating nature of this system averts unnecessary disposal of expensive isotope.

Growth containers for a hydroponic system are made of an inert material such as plastic (e.g., high-density polyethylene) or glass. Plastic containers have the advantage that they can be easily modified (drilling, cutting) to accept

plumbing connections, and thus can be readily interconnected to form a larger system. The containers, lids, and plumbing must be opaque, or should be covered with white-on-black plastic to occlude all light; penetration of light to the nutrient solution can promote algal growth and result in the diversion of isotope away from the intended plant. Covering containers with black plastic can lead to overheating of the nutrient solution and plant roots, unless the plants are housed in an adequately climate-controlled environment. Adequate size of the growth containers (depth and volume) is important, especially for those species that develop deep root systems.

Specific nutrient requirements vary for each plant species; however, some basic nutrient formulations have been developed for general plant growth.[12,24] Recent improvements to these formulations have focused on the maintenance of micronutrient metals in solution. A number of synthetic chelators are now commonly used to prevent metal precipitation, especially for Fe or Zn.[25–28] For recirculating systems, a starting formulation is chosen and then nutrients are replenished, either from concentrated stock solutions or from a refill solution, the composition of which may vary with plant developmental age. Nutrient levels are monitored frequently throughout plant growth to determine which nutrients must be replenished; techniques can range from inductively coupled plasma emission mass spectrometry, atomic absorption spectroscopy, and ion-selective electrodes, to colorimetric test strips. In cases of the simpler static system, a nutrient formulation is chosen, which must be replaced with fresh solution at varying intervals; nutrient concentrations still must be measured periodically to determine how often to replenish the solution. For either hydroponic approach, stable isotopes (low-abundance) are added to the system at an appropriate stage of development, either by mixing them into the replacement or refill solution in place of the normal high-abundance isotope(s), or by direct addition to the recirculating solution.

Besides mineral nutrients, two other concerns must be addressed in all hydroponic solutions: pH and oxygen. Nutrient solutions have only limited buffering capacity, relative to soils, and wide swings in pH are possible in response to the influx of various ions, especially ammonium and nitrate.[29] The pH of small-volume static systems can be controlled with the addition of one of the Good buffers, such as MES,[30,31] but large-volume recirculating systems are generally regulated with an in-line pH electrode connected to an electronic pH controller and metering pump for acid or base addition to the system. Plant root function is optimal at a pH between 5 and 7.[32] More acidic conditions lead to root dysfunction, necrosis, and nutrient influx imbalances, whereas alkaline conditions lead to precipitation of some cations. Oxygen is also an important variable, as it is needed to support respiration in the root zone. A stagnant solution containing roots can be depleted of oxygen quite rapidly, so oxygen must be added to solutions at all times. In static systems, this is usually accomplished by an air pump, which bubbles air into the solution through either a plastic tube or a sintered glass airstone. In recirculating systems, bubblers also can be used, but recirculating systems have an additional advantage in that air bubbles are generated in the nutrient solution as this solution cascades down from growth containers to the reservoir tank. A sufficient flow rate from the solution pump in a recirculating system is thus quite important to ensure adequate replenishment of oxygen (and other nutrients) throughout the growth containers.[21,33]

Stem injection

For mineral labeling, the injection of isotope into the stem is a technique which can maximize isotope partitioning to the harvested tissues (usually seeds).[20] Root absorptive and adsorptive processes are bypassed such that larger quantities of isotope can be introduced into the plant. Injections performed at upper-stem locations limit the amount of isotope being carried and possibly lost to the nonharvested vegetative tissues, via transpirational flow in the xylem stream. Stem injection, however, presents some potential problems. Because this technique involves a nonphysiological route of mineral introduction, the forms or amounts of the mineral transported in the stem pathway may not reflect the endogenous situation, nor may deposition in the harvested tissues reflect the endogenous situation. In the worst case scenario, if the injected dose grossly exceeds the transport or storage capacity of the plant, toxicities could be induced in reproductive organs, leaves, or at the point of application.[34] For these reasons, this technique is recommended primarily for micronutrient metals, and care must be taken in the quantities of isotope administered to the plant.

The form of the deposited isotope after stem injection has been studied for certain metals. The association of Zn or Cu isotopes with protein fractions separated from wheat grains (by gel filtration chromatography) was similar between stem-injected and hydroponic or control plants.[35,36] Protein fractions were not purified further to show specific metal-protein associations, but the results suggest that these isotopes were deposited in their natural forms. This would seem to be the case at least for Zn, because Zn radioisotope administration to excised wheat spikelets shows rapid uptake into the phloem pathway and movement to the grains.[37] Stem injection also has been used to label corn with Fe,[20] wheat grains with Ca,[10] and peanuts with Cu.[38]

The technique of stem injection uses a hypodermic needle and syringe to administer the isotope.[20,35] The needle is used to create an opening into the central pith region of the stem, or in some cases the hollow center. For hollow stems, a second opening can be made further up the internode to release the gas volume that will be displaced by the injected isotope solution. For solid stems, a plug of stem tissue should be removed to create a space to administer the isotope solution; this opening then can be covered with lanolin to prevent the solution and the exposed stem tissues from drying. Plants for this technique can be grown hydroponically or in soil.

The basis of this technique is that the isotope either diffuses into the xylem pathway and is carried up the plant via transpirational flow to the tissues of interest, or it diffuses to and is transported across the sieve element membrane for transport within the phloem pathway. Xylem-to-phloem exchange[39] also could occur further up the stem. Thus, the chemical form and the quantity of isotope admin-

istered are important for the success of this technique. Unfortunately, our knowledge of the endogenous transport of mineral ions in the xylem or phloem pathways is incomplete. We do know that various mineral cations either move as free ions or are chelated to organic acids or amino acids in the xylem pathway; anions generally move as free ions.[23,40,41] Little is known about the forms of minerals transported in the phloem pathway,[39] although evidence indicates that Fe must be chelated with an endogenous compound before entry into the phloem system.[42]

Atmospheric labeling

The labeling of all organic molecules can be accomplished through the introduction of $^{13}CO_2$ to the plant, because carbon fixed in the photosynthetic processes is ultimately passed on to all biosynthetic pathways. Labeling with CO_2 is a much more complicated endeavor than that of minerals, however, as it requires specialized equipment for gas containment, handling, and analysis. Undoubtedly, this complexity has turned many investigators away from this method, even though the demand for ^{13}C-labeled compounds is apparently high, as evidenced by the commercial availability of labeled sugars, lipids, and amino acids. Unicellular algae, rather than higher plants, usually are labeled to extract these compounds. Whereas these nutrients are useful for certain investigations, it is difficult to study the bioavailability of complex organic nutrients or phytochemicals without labeled plant foods. Multicomponent CO_2-labeling systems have been developed previously, but simplified approaches also are possible for certain applications.

The primary requirement of a $^{13}CO_2$-labeling system is a closed, relatively gas-tight chamber to house the plants and to isolate the enriched atmosphere. Because of the high cost of $^{13}CO_2$, the system should be of a closed loop design rather than an open, one-pass system. External light should be provided to the chamber, and because of the heat load this creates, the temperature and humidity of the internal air space must be regulated. Carbon dioxide concentration must also be regulated, and for long-term labelings, the oxygen concentration will need to be monitored and possibly adjusted. Plants should be grown in a hydroponic system within the chamber to prevent $^{12}CO_2$ release from carbonates in soils. At least four designs meeting these general specifications have been reported for labeling either with $^{14}CO_2$ or $^{13}CO_2$.[43–46]

The possible approaches to labeling plants with isotopic CO_2 can vary from continuous to short-term labeling, as well as from intact plants to excised leaves. The approach used will depend on the level of enrichment required, the tissue to be harvested, and the turnover rate of the compound of interest. For instance, if extremely high ^{13}C-enrichments are desired, intact plants may need to be grown continuously from germination to harvest within the labeling chamber. This would be the case especially if a reproductive organ, rather than a leaf, is the required food source. Likewise, if the compound to be studied has a low turnover rate and/or is degraded very slowly, an extended period of labeling may be needed to generate sufficient quantities of the compound.

For compounds with high turnover rates, or when low enrichments of the total pool of that compound are required, labelings can be done for a short duration using simpler systems. One example is the field labeling of rice plants to generate ^{13}C-labeled grains.[47,48] The system consists of an acrylic plastic enclosure (transparent) placed over a group of plants; water in the flooded field helps seal the bottom of the system. The enclosure contains only a fan to mix the airspace and a sealable port to allow the investigator to add $^{13}CO_2$ to the system and to sample the airspace (T.W. Boutton, personal communication). Gas samples are analyzed with an infrared gas analyzer to determine when additional CO_2 is needed. Another approach is to merely label excised leaves, whose petioles are placed in nutrient solution.[50] Plants can be grown in soil until the leaves are excised; these can then be placed in a transparent chamber for short-term labelings. This approach is useful obviously only for high-turnover compounds that are normally consumed in vegetative tissues.

General considerations

Plant selection

The type of plant to be labeled usually is predetermined by the investigator, based on the fact that it is the source of a particular nutrient and/or that the study is focused on a certain fruit, vegetable, or seed crop. Attention should be paid, however, to the specific cultivar chosen for labeling. Issues to consider are plant determinateness, plant size, and nutrient concentration. Plant determinateness refers to whether the growth habit of a plant is determinate (reproductive development occurs at terminal axes over a finite time frame and after vegetative growth) or indeterminate (vegetative organs continue to develop during reproductive growth). When the labeling of reproductive tissues is desired, determinate cultivars should be selected, as it is preferable to have all the pods, seeds, etc. at a near-uniform stage of development when the isotope is administered.

Plant size is an issue, in that it has relevance to the available space within the growth environment and also to the potential loss of isotope to non-harvested tissues. Semidwarf, high-yielding cultivars have been developed for a number of plants;[51,52] these are more amenable to the limited headspace of growth chambers or CO_2-labeling chambers. These short-statured varieties sometimes have reduced vegetative surface area such that for whole-plant labelings via hydroponic or atmospheric systems, the recovery of applied isotope may be increased in reproductive tissues.

Genetic variation for nutrient concentration has been reported for certain plant foods,[53,54] and it is likely that more variation in other crops will be identified as research in this area continues.[55] It is worth searching the horticultural and agronomic journals for information on nutrient-dense cultivars before making a selection. Cultivars with high rates of nutrient partitioning to the edible tissues theoretically can be labeled more efficiently and with higher recovery of administered isotope, thus lowering costs. Note also that if serving size is a concern, such as with small

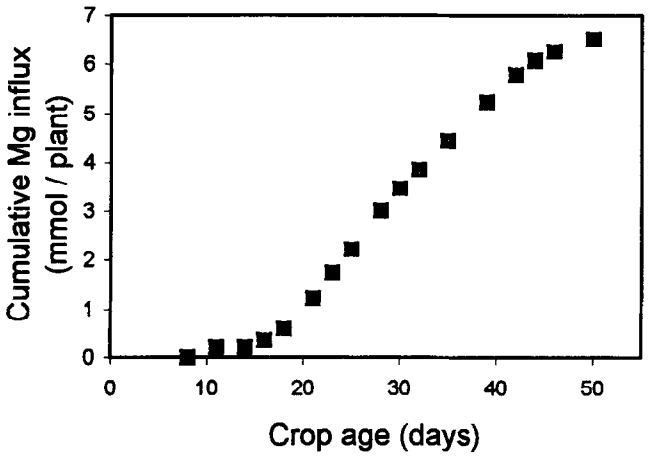

Figure 1 Cumulative Mg influx per plant for a hydroponically grown green bean crop. Planting density was 20 plants m^{-2}. Pods began growth on day 35 and reached harvest size by day 50. Plants were grown and nutrient flux was analyzed as described in Ref. 16.

Table 2 Sample calculations for determining isotope needs for a plant labeling to study Mg bioavailability in children

Study Specifications
A. Subjects: 13
B. Required dose: 8.5 mg ^{26}Mg/subject
C. Preferred serving size: 90 g fresh weight pods/subject
Measurements
D. Plant yield: 100 g fresh weight pods/plant
E. Pod Mg concentration: 0.17 mg/g fresh weight
F. Mg needs during labeling period: 5.27 mmol/plant
G. Isotope recovery: 12%
Calculations
H. Mg content of serving size: C × E = 15.3 mg*
I. Total pod weight needed: A × C = 1170 g fresh weight pods
J. Total plants needed: I ÷ D = 12 plants
K. Total Mg needed for labeling period: F × J = 63.24 mmol
L. ^{26}Mg needed for subjects: A × B = 110.5 mg
M. ^{26}Mg needed for plant labeling: L ÷ G = 921 mg (35.42 mmol)

*Note that the Mg content of this serving size is sufficient to achieve the required dose (B). Green bean plants are to be hydroponically labeled with ^{26}Mg; labeling will occur from day 21 through harvest at day 50.

children, nutrient-dense foods enable a required isotope dose to be given in a smaller quantity of food.

Growth parameters

The environmental parameters (temperature, humidity, light intensity and photoperiod) needed to achieve optimal growth vary among plant species and can vary among cultivars of the same species. The limits of these parameters for a given plant must be identified and adhered to for successful growth. Programmable environmental growth chambers thus are ideal for labeling studies, but even greenhouses can provide the necessary level of environmental regulation. Light intensity is important for vigorous growth, and the proper photoperiod (light/dark cycle) is a critical signal for some plants to proceed from vegetative to reproductive development.[56] If total darkness at night cannot be ensured in the growth environment, a photoperiod-insensitive (day-neutral) cultivar sometimes can be chosen.

Preparation for isotope labeling

Before labeling can begin, various measurements must be made on a group of test plants grown under the environmental conditions to be used by the investigator. These determinations include a measure of total plant uptake of the element of interest (mineral ions or carbon) before harvest, the yield per plant of the edible tissue, and an average concentration of the element in the harvested tissue. The approach we have taken with mineral nutrients has been to measure daily net influx of a mineral throughout crop development using our hydroponic system.[19] This has enabled us to determine cumulative mineral needs for any period of development, such as during reproductive growth.[16] An example of cumulative Mg influx by a hydroponically grown green bean plant is shown in *Figure 1*. Similar information could be gathered by harvesting plants at different developmental stages and analyzing the mineral content of all tissues. For carbon accretion, sequentially harvested plants could be analyzed for total carbon content. Alternatively, net CO_2 exchange could be measured in intact plants or excised leaves at various stages of crop development using an infrared gas analyzer and gas flow meter.[57]

Once these values are known, an estimate of the anticipated isotope recovery in the edible tissues must be made for a particular labeling approach. This could be based on the mineral or carbon analyses of harvested tissues, or a test labeling could be performed on a limited number of plants (possibly using a radioisotope instead of a stable isotope). Then, for a preferred serving size and a required isotope dose per subject (a function of the natural abundance of the chosen isotope and the endogenous levels of that nutrient in the test subject),[5,58] the feasibility of achieving that dose in said serving size can be determined from the nutrient concentration of the tissue. Finally, for a given number of subjects, the quantity of isotope needed for the labeling and the number of plants to be labeled can be calculated as shown in *Table 2*.

Timing and site of isotope application

The transport systems used by a plant to deliver nutrients to an organ of interest will determine when and where isotope should be administered. Long-distance nutrient transport occurs within either the xylem or phloem pathways.[22] The xylem system carries water and nutrient solutes (minerals, amino acids, secondary metabolites) from the roots to all transpiring organs, with this movement being driven principally by the gradient in water potential between roots and shoots. Total xylem-associated nutrient import by an organ will depend on cumulative transpiration from that organ and the concentration of nutrients in the xylem fluid. The phloem system is a longitudinal network of membrane-bound living cells that transports nutrients primarily from photosynthesizing organs (source tissues) to immature leaves, developing reproductive tissues, and the root system (sink tissues). Transport is driven by a source-to-sink hydrostatic gradient that is generated by the influx (phloem loading) and the efflux (phloem unloading) of major solutes

into/from the phloem of source and sink tissues, respectively. The phloem-associated flow of nutrients (sugars, amino acids, minerals, secondary metabolites) to an organ will depend on their capacity for phloem loading, their rate of loading, and the volume flow of phloem sap from their loading site to the sink organ. In the case of minerals, note that some are poorly phloem loaded and thus have limited phloem mobility.[22]

To effectively label a plant tissue, attention must be paid to the primary pathway of import for a nutrient and where that nutrient enters the transport system. For nutrients first localized in the root zone, the primary nutrient flow to edible shoot organs, such as leaves (spinach, kale), immature floral organs (broccoli, cauliflower), or pods (green beans, snow peas), occurs in the xylem pathway. This is the case not only for minerals absorbed at the root surface, but also for amino acids and secondary metabolites that are synthesized in the root tissues[59] and subsequently enter the xylem pathway. Thus, hydroponic culture or stem injection would be appropriate approaches for intrinsic isotope enrichment of xylem-fed tissues, with hydroponic culture required for the labeling of root-synthesized compounds and recommended for the labeling of leafy vegetables.

In the case of predominantly or exclusively phloem-fed tissues (seeds, fruits), introduction of the isotope must be tailored to ensure that it enters a source region capable of loading the nutrient into the phloem pathway and with the appropriate vascular connections to carry the nutrient to the terminal tissue of interest. For instance, Fe transport to pea seeds occurs exclusively via phloem transport, with phloem Fe loading occurring within all leafy structures as well as within the pod walls.[42] Hydroponic introduction of Fe isotope to the plant will deliver Fe to all source regions via xylem transport, where it then can enter the phloem system for movement to the seeds. Stem injection of Fe, even to only the peduncle leading to the pod, also would be an acceptable approach, because the pod walls can phloem-load Fe. With respect to organic compounds that are synthesized in leaves and subsequently phloem-transported, $^{13}CO_2$ should be introduced into source leaves that efficiently synthesize the compound of interest, and that are direct source regions for the edible tissues to be harvested.

Future labeling possibilities

To date, most of the studies using intrinsically-labeled foods have focused on issues of mineral bioavailability. Clearly, however, there are various vitamins, unique phytochemicals, and other complex compounds of dietary significance, the absorption and metabolism of which beg to be studied. Because many of these compounds are composed predominantly of carbon and hydrogen, there may be a reluctance to attempt to label them, especially because of the difficulties inherent in $^{13}CO_2$ labeling. There are, however, alternative approaches that may be considered.

Labeling with heavy water

Plants obtain almost all of their hydrogen from water, either directly, after the splitting of absorbed water in the photosynthetic reactions, or indirectly, when protons dissociated from water are absorbed by root membrane transport systems. This hydrogen is then incorporated into all hydrogen-containing molecules throughout the plant. Heavy water (deuterium oxide, D_2O), a rather inexpensive isotopic molecule, could provide a cost-effective means for introducing a stable hydrogen isotope into the plant. Labeling could be based on hydroponic culture, in which a portion of the H_2O in the nutrient solution is replaced with D_2O. All water entering the plant would be carried in the xylem stream to each transpiring organ. If plants are grown continuously using the supplemented solution, the deuterium enrichment of the plant and all of its compounds should reflect the enrichment of the nutrient solution.[60] It should be noted, however, that water transport in plants involves not just root absorption, but also transpiration from shoot structures. This means that a good portion of the administered heavy water merely will move through the plant and be lost to the atmosphere. This loss could be lowered by growing the plants at high relative humidity, thus reducing the gradient for transpiration, or by growing the plants in an enclosure in which the outgoing airstream is passed through a condensing system to recover the D_2O.

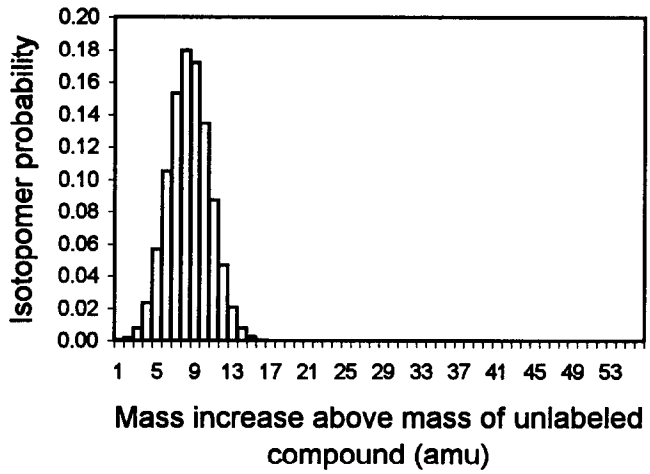

Figure 2 Predicted isotopomer profile for deuterium-labeled β-carotene extracted from a plant cultured on 15 atom % heavy water.

The level of deuterium enrichment one might attempt will depend on the chemical composition of the compound of interest, as well as the technical resolution available to discern certain isotopomers of the labeled compound.[61] For instance, the provitamin A carotenoid, β-carotene, has the chemical formula $C_{40}H_{56}$. By taking into account the probability of a deuterium replacing a hydrogen at various positions along the molecule, in addition to the number of possible specific structures for each isotopomer, a 15-atom % deuterium labeling of a plant should yield an isotopomer profile for β-carotene similar to that shown in *Figure 2*, with a peak in the M+8 isotopomer pool (note that the natural abundance of ^{13}C was not factored into this prediction). Thus, a labeling of this sort would facilitate the mass spectrometric determination in plasma of both the M+8 isotopomer of β-carotene and the M+4 isotopomer of retinol, derived from the ingested β-carotene.[62]

What is the feasibility of labeling a plant to 15 atom %

deuterium? Although the administration of 20 to 30 atom % D_2O to mice or dogs leads to serious organ malformations and sterility,[63,64] higher plants can be labeled to as much as 30 atom % deuterium with no significant effect on growth or development, and to 50 atom % deuterium with only a moderate decline in growth.[60] Above 60 atom % D_2O, there is a significant decline in plant growth, as well as an inhibition of reproductive development. Interestingly, unicellular algae have been successfully grown on 99.6 atom % D_2O.[65]

Hairy root cultures

Plant roots are incredible biosynthetic factories, responsible for the production of alkaloids, sesquiterpenes, polyacetylenes, and many plant secondary metabolites.[59] Roots usually grow in a darkened environment as heterotrophic organs, and depend on shoot tissues for their organic nutritional support. Culture techniques are available, however, for growing roots isolated from their shoots;[66,67] these roots can be grown in a medium supplemented with sucrose and essential vitamins, or for some species they may be induced to develop chloroplasts, and can achieve photoautotrophy when supplied with light and CO_2.[68] One type of culture system, the hairy root culture (so named for the proliferation of adventitious roots), is obtained by inoculating a plant stem with the bacterial pathogen *Agrobacterium rhizogenes*.[67,69] A bacterial plasmid containing root-inducing genes is transferred to and integrated into the genome of the infected plant cells; adventitious roots that develop from the infection site are removed and placed in a culture medium. These isolated roots can grow as rapidly as unorganized plant cell suspensions and can exhibit the endogenous biosynthetic pathways expressed in normal roots.[70,71] Because *Agrobacterium rhizogenes* also is used to generate transgenic plants, it should be noted that hairy root cultures potentially could be developed with the capacity to express an unlimited array of unique metabolites and proteins of both nutritional and therapeutic value.

The ability to maintain a photoautotrophic root culture raises the possibility of labeling roots with $^{13}CO_2$ in a simple bioreactor system to generate labeled compounds. $^{13}CO_2$ could be bubbled into the culture solution from a gas supply, or a CO_2-generating buffer could be established to provide a $^{13}CO_2$ environment.[68] Alternatively, a heterotrophic culture of hairy or isolated roots could be supplied with ^{13}C-labeled sucrose. While hairy roots are not a common food in the human diet, their use would provide a cost-effective method for labeling unique plant compounds that could be extracted and administered extrinsically. Although this is not an ideal method to study bioavailability, it at least could provide a first attempt to assess absorption potential in the gut, before proceeding to more complicated whole-plant labeling methodologies.

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