VARIATIONS IN STABLE CARBON ISOTOPE VALUES OF DIETARY BIOCHEMICALS: EXTRACTION PROCEDURES AND RESULTS

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ABSTRACT

It is now known that the ratio of ^{13}C:^{12}C differs between bulk plant material and its individual macronutrients. It is necessary to isolate and purify each biochemical without altering its isotopic signal to accurately assess the contributions of individual macronutrients to a bulk diet. We modified and enhanced published procedures for the isolation and purification of five basic fractions in plant material. Lipids were dissolved in chloroform:methanol and the solvent evaporated. Proteins were isolated by solubilizing in dilute NaOH, then salting out with saturated ammonium sulfate. Cellulose and lignin were purified by Goering and Van Soest's method of sequential detergent extraction. Starch was solubilized in DMSO and then precipitated with ethanol. With the exception of starch, we were able to extract individual unfractionated biochemicals from a known test mixture. Mass balance calculations from individual biochemicals reconstructed the bulk value with high precision.

We used these procedures to analyze live and dead grasses, modern and ancient corn seed, and wheat seed. Lipids were consistently the most depleted biochemical relative to bulk material. Lignin was depleted 2 to 5‰ and protein was 1 to 3‰ more negative than the bulk. In grasses, cellulose was 0 to 3‰ more positive, whereas in corn and wheat seeds, it was 0 to 3‰ more negative. Starch was always enriched 0.5 to 2‰ compared to bulk seed material.

INTRODUCTION

The analysis of stable carbon isotopes offers a unique tool for tracing the path of carbon through food webs. Two photosynthetic pathways known as C_3 and C_4 provide for differential fixation of atmospheric ^{12}C and ^{13}C. This results in a distinct isotopic label that can be measured and subsequently traced from diet to consumer. The effective transfer of this label from one trophic level to another provides the basis for paleodietary reconstruction (DeNiro, 1989; DeNiro and Epstein, 1978) and other dietary studies (Tieszen et al. 1979).

We now know, however, that different animal tissues possess slightly different isotopic signatures (Tieszen et al., 1983) and that the diet collagen spacing in herbivores is greater than in carnivores. A current model suggests that in herbivores there is a 5‰ fractionation between bone collagen and bulk diet (Krueger, 1984). The same model suggests that in carnivores there is no isotopic variation between collagen and diet. To accurately reconstruct what an organism has been eating, it is essential to understand the relationship between the isotopic value of the tissue being analyzed and the isotopic values of the dietary inputs.

It is likely that collagen is synthesized not from a mixture of all foods ingested, but from specific macronutrients in those bulk materials. If the isotopic value in a particular macronutrient differs from the whole diet, that difference may be assimilated into specific tissues. For instance, if dietary protein is depleted in ^{13}C, collagen synthesized from protein may also be depleted. If an animal eats a low protein diet, none of its amino acids may be synthesized *de novo*, thereby altering the isotopic composition. It therefore becomes necessary to determine variations in the isotopic composition of macronutrients, their concentrations in certain feeds, and the digestibility of those macronutrients in order to better understand how isotopes in diet relate to isotopic signals in tissue.

To characterize the range of isotope values in macronutrients, extraction procedures which isolate biochemicals from bulk material without altering their isotopic signal are necessary. We modified and tested procedures for extracting lipid, cellulose, lignin, protein, and starch from dietary material. Analyses was then performed on various types of maize, grass, and wheat.

MATERIALS AND METHODS

Figures 1-3 describe the flow charts of the extraction procedures. The appendix documents the details of solution preparation. A ground 3 gram sample was used to extract lipid, cellulose, and lignin. The sample was first placed in 35 ml of chloroform:methanol (2:1), homogenized in a blender for 20 seconds, and filtered through a sintered glass funnel. The solid residue was saved for cellulose extraction. Five ml of 0.2 M KCl was added to the filtrate. This solution was centrifuged at 12,000 x *g* for 10 minutes and the top phase discarded. The lower organic phase was washed with an equal volume of methanol: 0.2 M KCl (1:1). After another centrifugation at 12,000 x *g* for 10 minutes, the top phase was discarded. A second wash was performed to remove any residual non-lipid material. The methanol:chloroform solvent was finally allowed to evaporate, leaving a lipid residue (Figure 1 -- Enterman, 1957).

A method of sequential detergent analysis was used to isolate cellulose and lignin. The pellet from the final centrifugation in the lipid extraction was refluxed with 100 ml neutral detergent solution (NDS) and 2 ml of decahydronaphthalene. NDS was made by dissolving 18.61 g disodium ethylenediaminetetraacetate (EDTA) and 6.81 g sodium borate decahydrate in 100 ml distilled water and then dissolving this in a solution containing 30 g sodium lauryl sulfate and 10 ml 2-ethoxyethanol (ethylene glycol monoethyl ether). Next, a solution containing 4.56 g disodium hydrogen phosphate was added and the entire mixture was made up to 1 liter with distilled water. After refluxing the sample for one hour, the solution was filtered and rinsed with hot water and acetone. Pectins, tannins, silica, and starch were dissolved by the hot neutral detergent. The solid residue was then refluxed with 100 ml acid detergent solution and 2 ml of decahydronaphthalene for another hour. ADS was made by combining 20 g cetyl trimethylammonium bromide (CTAB) and 27.7 ml conc. sulfuric acid in 1 liter. Once again, the reflux solution was filtered and rinsed with hot water and acetone. Proteins and hemicellulose were dissolved in this process. What was left was lignin, cellulose, and some cutin.

One half of the residue was placed in a büchner filter funnel. Cooled 72% sulfuric acid was added over a three hour period. After polysaccharides were dissolved, the remaining lignin residue was rinsed to neutrality with distilled water (Figure 1 -- Goering and Van Soest, 1970).

The remaining acid detergent fiber was used to isolate cellulose. Lignin was oxidized using an excess of acetic acid-buffered potassium permanganate solution, containing trivalent iron and monovalent silver as catalysts. The potassium permanganate solution was made with 50.0 g potassium permanganate and 0.05 g silver sulfate dissolved in 1 liter of distilled water. A lignin buffer solution of 6.0 g ferric nitrate nonahydrate and 0.15 g silver nitrate dissolved in 100 ml of water, 500 ml acetic acid, 5.0 g potassium acetate, and 400 ml tertiary butyl alcohol was also prepared. The final potassium permanganate solution consisted of 66.6 ml saturated potassium permanganate solution and 33.3 ml lignin buffer. After 90 minutes, manganese and iron oxides were dissolved with an alcoholic solution of oxalic and hydrochloric acids. This demineralizing solution consisted of 50 g oxalic acid dihydrate in 700 ml 95% ethanol and 50 ml conc. HCl in 250 ml distilled water. The final residue was washed with ethanol and acetone (Figure 1 -- Goering and Van Soest, 1970).

We tried two main approaches for isolating protein. The first involved soaking a two gram sample in a NaOH solution with a pH of 11.0. After five hours, the solution was centrifuged at 12,000 x *g*

EXTRACTION OF LIPID*, CELLULOSE**, AND LIGNIN**

3 g ground sample
Add 35 ml chloroform:methanol (2:1)
Homoginize 20 seconds in a blender
Filter through a sintered glass funnel

Solid
place in 500 ml reflux flask
Add 100 ml cold NDS†
Add 2 ml decahydronaphthalene
refux 60 min
filter in Gooch crucible
wash mat with hot water
wash mat with acetone
suck dry

Solid
place in 500 ml reflux flask
Add 100 ml ADS†
Add 2 ml decahydronaphthalene
reflux 60 min
filter in Gooch crucible
wash mat with hot water
wash with acetone, suck dry
(divide into two fractions)

Solid #1
place in a Gooch crucible in a shallow pan filled with 1 cm distilled water
Add 25 ml combined permanganate solution†
let stand 90 minutes
add more solution as needed to keep purple
suck crucible dry, put in clean pan
Add demineralizing solution† to crucible
treat until residue is white
wash twice with 80% ethanol
wash twice with acetone
suck dry
residue is CELLULOSE

Solid #2
place on glass filter paper
place in a buchner filter funnel
Add cooled 72% sulfuric acid
let acid slowly drip through
let sit 3 hours, adding acid as needed
drain off acid
wash with distilled water until neutral
residue is LIGNIN

Filtrate
Add 5 ml 0.2 M KCl and mix
Centrifuge 10 min @ 12,000x*g*
Pipet off top phase and discard

Wash with 10 ml methanol:KCl (1:1)
Centrifuge 10 min @ 12,000x*g*
Pipet off top phase and discard
Repeat wash
Evaporate solvant and lypholize to dryness
Residue is LIPID

*Enterman 1957
**Goering and VanSoest 1970

Figure 1. Flow chart for the extraction of isotopically intact lipid, cellulose, and lignin.

EXTRACTION OF PROTEIN*

3 g ground sample
Add 30 ml chloroform:methanol (2:1)
Homoginize 15 seconds in a blender
Centrifuge 10 min @ 12,000x*g*

pellet — supernatant (discard)

Dry at 60°C for 30 min
Add 30 ml cold 0.025 M NaOH and mix
Centrifuge 10 min @ 12,000x*g*

pellet — supernatant

Add 10 ml 70% $(NH_4)_2SO_4$, pH 4.0
Boil for 10 minutes
Centrifuge 10 min @ 12,000x*g*

pellet — supernatant

Wash pellet twice with chloroform
Wash pellet twice with acetone
Lypholize to dryness
residue is PROTEIN

*Robinson 1980

Figure 2. Flow chart for the extraction of isotopically intact protein.

EXTRACTION OF STARCH*

1 g ground sample
place in a 30 ml centrifuge tube
Add 30 ml 90% dimethyl sulfoxide (DMSO)
place on a shaker for 48 hours
centrifuge @ 480x*g* for 5 minutes

pellet — supernatant

discard

add 20 ml of cold 95% ethanol
chill at 4° C for 12 hours
centrifuge @ 12,000x*g* for 5 minutes

pellet — supernatant (discard)

add 30 ml of cold 95% ethanol
mix well
centrifuge @ 12,000x*g* for 5 minutes

pellet — supernatant (discard)

residue is STARCH

*Brown & Volenec 1989

Figure 3. Flow chart for the extraction of isotopically intact starch.

for ten minutes. The pH of the supernatant was then lowered to 4.5 with HCl. Protein was precipitated by first boiling the solution for two minutes and then centrifuging at 12,000 x *g* for ten minutes (Winkler et al., 1978).

The method we finally adopted results in the purification of glutelins. Prolamines, albumins, globulins and histones were assumed to constitute a small percentage of total protein in corn seed. Lipid was first removed by adding 30 ml of chloroform:methanol (2:1) and homogenizing 15 seconds in a blender. This mixture was centrifuged at 12,000 x *g* for ten minutes, and the solvent decanted. After the pellet thoroughly dried in an oven, 30 ml cold 0.05 M sodium hydroxide was added and allowed to soak for 30 minutes. Non-soluble material was removed during a ten minute centrifugation at 12,000 x *g*. Ten ml of 70% ammonium sulfate, pH 4.0 was added to the supernatant. After boiling 10 minutes, a final centrifugation at 12,000 x *g* precipitated the protein. The pellet of protein was washed with chloroform and acetone and finally dried (Figure 2 -- Robinson, 1980).

Each of the three starch extraction procedures we tried resulted in altered carbon isotope values. The method which produced the more accurate results was adopted from Brown & Volenec (1989). A 1 gram sample was ground and placed in a 30 ml centrifuge tube filled with 90% (v/v) dimethyl sulfoxide (DMSO). After agitating 48 hours, the suspensions were centrifuged 5 minutes at 480 x *g*. Starch in the supernatant was precipitated with 20 ml cold 95% (v/v) ethanol and chilled for 12 hours. The starch precipitate was then centrifuged at 12,000 x *g* for 5 minutes. After discarding the supernatant, 30 ml cold 95% ethanol was added to the pellets. Following recentrifugation, the ethanol supernatant was discarded and the pellet was allowed to dry. The main problem with this method is that DMSO is also a good solvent for protein. To counter this, we attempted to wash the final pellet in 0.05 M NaOH to remove protein (Figure 3).

Another method we tried involved boiling a 1 g sample in ammonium carbonate for 30 minutes, centrifuging, and complexing the starch in the supernatant with iodine-potassium iodide. The insoluble iodine-starch was then centrifuged out and disassociated with sodium thiosulfate. The final starch was washed first with hydrochloric acid, then by ethanol (Steiner and Guthrie, 1955).

A third procedure utilized 10% trichloroacetic acid to dissolve starch. After centrifuging, starch was reprecipitated with the addition of 2 vol ethanol. The final residue was washed several times with ethanol and dried (Prescott, 1965).

A Cahn microbalance was used to load between 1.5 and 2.0 mg of each sample into a tin capsule. These cups were then combusted in a Carlo Erba 1500 nitrogen/carbon analyzer. The resultant gases were chromatographically separated, quantified and routed through a SIRA series II isotope ratio mass spectrometer from which $\delta^{13}C$ values relative to PDB were obtained. Our mass spectrometer is able to produce results with an accuracy of $0.2^{o}/_{oo}$. Percent nitrogen and percent carbon in each sample were calculated.

RESULTS AND DISCUSSION

Three approaches were used to verify that our extraction procedures were not altering the isotopic ratio. The first involved using the methods outlined above to extract individual biochemicals whose isotope value was known. The four pure biochemicals we used were Sigma corn starch (no. S-4126), Sigma wheat gluten (no. G-5004), Teklad non-nutritive cellulose fiber, and soy oil from Zeigler Bros. Inc. The isotopic values we obtained from these four biochemicals after extraction were not statistically different from the original unextracted macronutrients. This shows that our procedures do not, in themselves, alter the isotopic signal (Table 1).

Next, we physically mixed the original pure starch, cellulose, protein, and lipid together in a 1:1:1:0.3 ratio. Each macronutrient was then extracted from the mixture (Table 1). With the exception of starch, the values we obtained were statistically the same as those from original unextracted biochemicals. As our method of starch extraction did not alter its isotopic value, we infered that one of the other three biochemicals was contaminating the starch sample. To test this, we created three more mixtures consisting of a 1:1 ratio of starch:cellulose, starch:protein, and starch:lipid (Table 2). After extracting starch from these mixtures, we found that starch from the starch:protein set departed the most from the pure starch value. We thus assume that protein is the main contaminate of our starch extracts and alters the starch isotope value. The other two starch extraction procedures we attempted produced results which were more unacceptable than the procedure using DMSO (Table 2).

As a final test, we performed a mass balance calculation on the extractions from both purified macronutrients and bulk forage material. We summed the products of the percent of a particular biochemical in each mixture or bulk material and the percent carbon in that biochemical and the isotopic value for that component. This number was then divided by the sum of the products of the percent biochemical in each mixture and the percent carbon in that biochemical. We assumed that cellulose contains 40% carbon and makes up 70% of grasses and 3.6% of corn seed. Starch we estimated to contain 40% carbon and to constitute 78.8% of corn seed. In both grass and corn, we assumed lipid contributes 5% of the weight and contains 71% carbon. Protein was estimated to contain 45% carbon and to make up

10-15% of plant material (Nakamura, 1982 and Benner 1987). By comparing the actual bulk isotopic value for each mixture to the weighted sum of the four main biochemicals we could discern whether or not our extraction procedure altered the $\delta^{13}C$ values. The mass balance we obtained from a mixture of pure unextracted chemicals was essentially identical to the measured isotopic value of the complete mixture (Table 3). When we compared calculated mass balances of grasses and corn seeds with the actual material we obtained a regression with an r^2 of 0.99 (Figure 4). This verifies that the values we place on the individual macronutrients in a naturally occurring bulk material reflect the true carbon isotope value.

Table 1. Percent composition of test biochemical, original carbon isotope values, and extracted values. A one-factor ANOVA was used to test if the carbon isotope value was altered by extraction.

BIOCHEMICAL COMPONENT	%C	%N	$\delta^{13}C$ (n=3)	Std. Error	F-test
LIPID		0.0	-29.78	0.14	2.16
Extraction from pure biochemical		0.0	-30.21	0.19	p=.186
Extraction from mixture		0.0	-29.99	0.10	
PROTEIN	46.0	13.7	-25.74	0.13	0.32
Extraction from pure biochemical	42.6	15.4	-25.73	0.14	p=.735
Extraction from mixture	43.0	14.6	-25.61	0.11	
CELLULOSE	30.9	0.0	-23.64	0.13	0.90
Extraction from pure biochemical	40.2	0.0	-23.79	0.14	p=.455
Extraction from mixture	38.2	0.0	-23.87	0.11	
STARCH	39.0	0.0	-10.46	0.16	36.05
Extraction from pure biochemical	38.0	0.0	-10.54	0.27	p=0.0002
Extraction from mixture	40.7	0.0	-13.66	0.38	

Table 2. Mean carbon isotope values for starch using various extraction procedures and mixtures.

	Extraction source				
Extraction procedure	pure starch	four-part mixture	starch+ lipid	starch+ cellulose	starch+ protein
Iodine complex	-11.71	-16.35			
Trichloroacetic acid	-12.09	-14.12			
DMSO	-10.54	-13.66	-11.13	-11.74	-14.12

Original carbon isotope value for starch is -10.46

Table 3. Calculation of mass balance based upon the original biochemicals and upon extraction from a mixture of those biochemicals.

			Carbon isotope	
Component	% in diet	% C	original	extracted
Lipid	9.1%	71%	-29.78	-29.99
Cellulose	30.3%	40%	-23.64	-23.87
Starch	30.3%	40%	-10.46	-13.66
Protein	30.3%	45%	-25.74	-25.61
Mass balance			**-21.58**	**-22.50**

Carbon isotope of bulk mixture was **-21.53**

Mass Balance =

(% Lipid in diet * % Carbon in Lipid * Carbon isotope value of Lipid +
% Cellulose in diet * % Carbon in Cellulose * Carbon isotope value of Cellulose +
% Starch in diet * % Carbon in Starch * Carbon isotope value of Starch +
% Protein in diet * % Carbon in Protein * Carbon isotope value of Protein)
/ (% Lipid in diet * % Carbon in Lipid +
% Cellulose in diet * % Carbon in Cellulose +
% Starch in diet * % Carbon in Starch +
% Protein in diet * % Carbon in Protein)

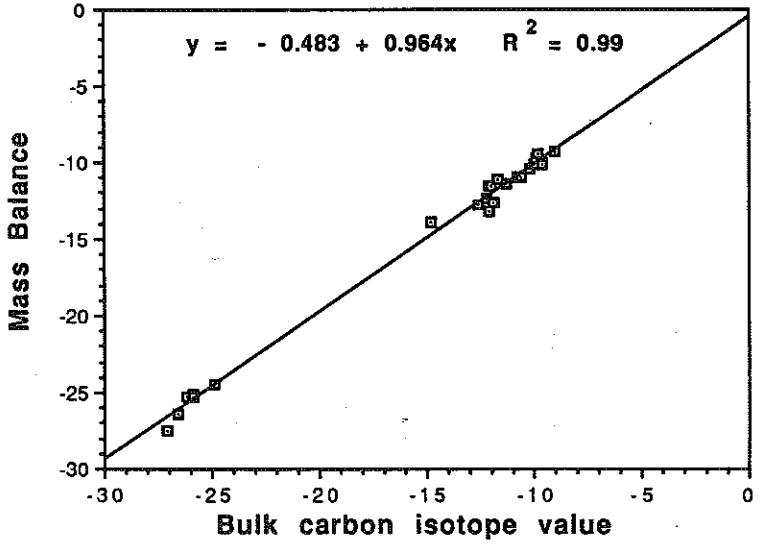

Figure 4. Regression of the calculated mass balance against the original plant or bulk isotope value.

Table 4. Carbon isotope values of maize seeds, grass leaves, extracted biochemicals, and the calculated mass balance.

CORN SEED	Cellulose	Starch	Lipid	Protein	Seed	Mass balance
Pioneer 6G39	-12.49	-10.69	-16.93	-12.89	-12.13	-11.51
Pioneer 8G84	-12.38	-10.17	-17.06	-12.48	-11.65	-11.07
Pioneer 8G86	-12.11	-10.05	-16.62	-13.24	-10.76	-11.02
CIMMYT 2267	-11.84	-8.43	-14.45	-12.23	-9.76	-9.48
CIMMYT 217	-12.75	-9.21	-14.65	-12.04	-9.98	-10.10
CIMMYT 1385	-13.38	-11.11	-15.66	-10.20	-12.00	-11.46
Ancient AZ 140 T-13	-12.97	-9.33	-14.86	-11.88	-9.58	-10.20
Ancient AZ 140 T-XPB3	-9.60	-9.43	-17.00	-11.99	-10.20	-10.35
Ancient AZ 140 T-131	-10.18	-8.52	-16.09	-13.53	-9.90	-9.77
Ancient AZ 140 T-94	-9.42	-8.92	-14.06		-9.00	-9.36
Ancient AZ 140 T-67	-9.55	-9.44	-16.33		-9.62	-10.02
% of biochemical in seed	3.6%	78.8%	4.7%	10.4%		
% of carbon in biochemical	40.0%	40.0%	71.0%	45.0%		

GRASS	Cellulose	Lignin	Lipid	Protein	Leaf	Mass balance
L. *Andropogon gerardi*	-10.27	-13.57	-17.60	-12.20	-11.34	-11.45
D. *Andropogon gerardi*	-11.58	-15.44	-18.37	-13.29	-12.61	-12.73
L. *Bromus inermis*						
D. *Bromus inermis*	-23.11	-27.46	-28.74	-26.06	-24.88	-24.39
L. *Calamagrostis canadensis*	-26.43	-29.18	-31.93	-29.24	-27.10	-27.56
D. *Calamagrostis canadensis*	-24.43	-29.70	-28.13		-25.87	-25.27
L. *Panicum virgatum*	-9.94	-12.56	-15.94	-12.06	-10.63	-10.99
D. *Panicum virgatum*	-11.32	-16.36	-19.58		-11.87	-12.61
L. *Sorghastrum nutans*						
D. *Sorghastrum nutans*	-11.91	-15.98	-19.25	-13.91	-12.07	-13.16
L. *Spartina pectinata*	-10.46	-20.34	-19.17	-13.32	-12.15	-12.39
D. *Spartina pectinata*	-12.14	-20.01	-21.87		-14.85	-13.83
L. *Stipa*	-24.21	-28.16	-30.76	-24.82	-25.91	-25.16
D. *Stipa*	-24.46	-28.49	-29.28		-26.22	-25.30
L. *Typha latifolia*						
D. *Typha latifolia*	-25.60	-28.69	-31.26		-26.61	-26.45
% of biochemical in leaf	70.0%	5.0%	5.0%	5.0%		
% of carbon in biochemical	40.0%	60.0%	71.0%	45.0%		

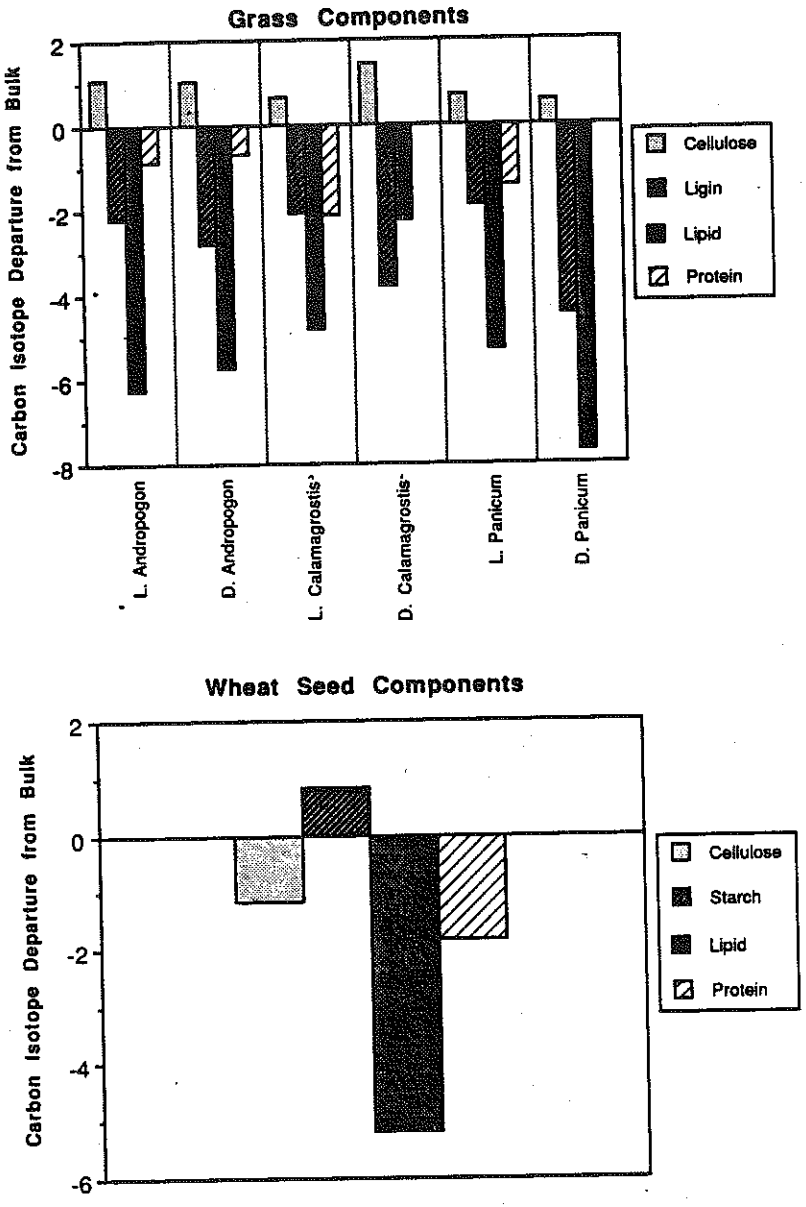

Figure 5. Isotopic departure of biochemical components for live and dead grass material and wheat seed.

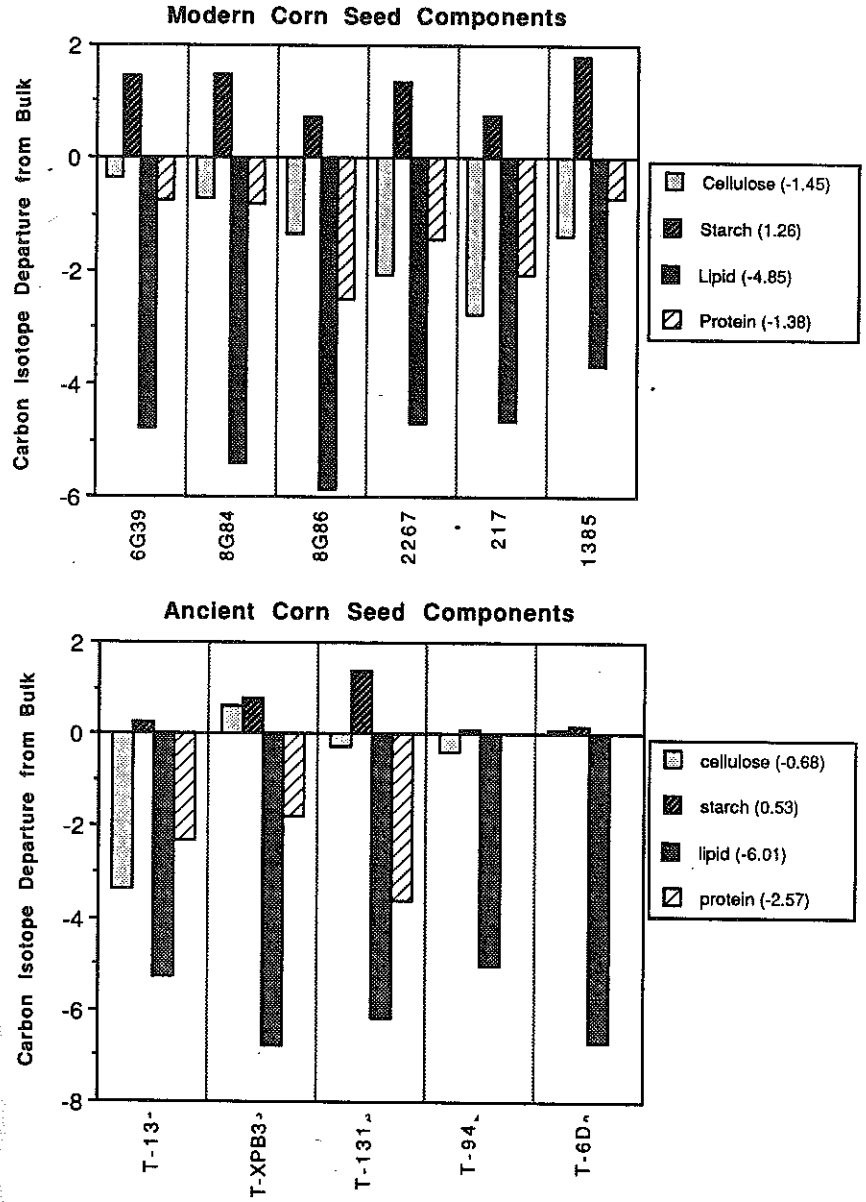

Figure 6. Isotopic departure of biochemical components for original maize seeds for modern and ancient seeds.

The general trends we observed from extracted macronutrients were consistent for each variety of maize and grass analyzed (Table 4). Lipid was depleted 5-6°/$_{oo}$, Lignin was depleted 2-5°/$_{oo}$, and protein was depleted 1-3°/$_{oo}$. As a large constituent in grasses, cellulose was either slightly enriched or closely resembled the bulk material. In maize, cellulose was slightly depleted 0-3°/$_{oo}$. Our estimates of starch indicate that it is always enriched 1-2°/$_{oo}$ in corn seeds (Figures 5 & 6). There is also a distinct difference between the bulk material of live and dead grasses. While this trend is not consistently more positive or negative, it does indicate there is isotopic alteration during decomposition.

There are distinct variations in the isotopic values in the macronutrients of bulk diets. There are also variations in the physical content of macronutrients in various feed material. Further investigation needs to be done on the role of each of these dietary biochemicals in the formation of tissues. Specific attention should be paid to the roles played in the formation of collagen. Reconstruction of paleodiets can then be greatly improved.

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APPENDIX

Protocol for preparation of materials and solutions

Neutral Detergent Solution (NDS). Mix 18.61g Disodium ethylenediaminetetraacetate (EDTA) and 6.81 g Sodium borate decahydrate in a large beaker, add some distilled water and heat until dissolved. Add this to a solution containing 30g Sodium lauryl sulfate and 10 ml 2-ethoxyethanol (ethylene glycol monoethyl ether). Put 4.56g Disodium hydrogen phosphate in a beaker with some distilled water, heat until dissolved. Mix in with the other solution. Make up final solution to 1 liter with distilled water. Check that the final pH is 7.

Acid Detergent Solution (ADS). Place 20g Cetyl trimethylammonium bromide (CTAB) in a 1 liter flask. Add 50 ml distilled water, 27.7 ml concentrated sulfuric acid and mix. Make up to 1 liter with dist. water.

72% Sulfuric Acid. 110 ml distilled water added to 890 ml concentrated sulfuric acid.

Saturated Potassium Permanganate. 50g Potassium permanganate and 0.05g Silver sulfate in 1 liter of water.

Lignin Buffer. Dissolve 6g Ferric nitrate nonahydrate and 0.15g silver nitrate in 100 ml distilled water. Add 500 ml acetic acid and 5g potassium acetate. Mix in 400 ml tertiary butyl alcohol.

Combined Permanganate Solution. Mix 66.6 ml saturated potassium permanganate + 33.3 ml lignin buffer. Keep these three solutions refrigerated. Discard if precipitate forms or if red color develops.

Demineralizing solution. Dissolve 50g Oxalic acid dihydrate in 700 ml 95% ethanol. Add 50 ml conc. HCl and 250 ml distilled water.