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The effect of chemical processing on the $\delta^{13}C$ value of plant tissue

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Abstract—The effect of standard processing techniques on the $\delta^{13}C$ value of plant tissue was tested using species representing the three photosynthetic pathways, including angiosperms and gymnosperms within the C_3 taxonomic division. The species include *Cowania mexicana* (C_3 angiosperm), *Juniperus osteosperma* (C_3 gymnosperm), *Opuntia* spp. (crassulacean acid metabolism [CAM] angiosperm), and *Atriplex canescens* (C_4 angiosperm). Each species is represented by 5 plants collected at two different sites, for a total of 10 samples. The samples were processed to whole plant tissue, holocellulose, α-cellulose, and nitrocellulose. An additional process was added with the discovery of residual Ca-oxalate crystals in holocellulose samples. Both C_3 species show $\delta^{13}C$ values becoming ^{13}C enriched with increased processing. The CAM representative shows the opposite trend, with ^{13}C depletion during the progression of treatments. The greatest range of values and most inconsistent trends occur in the C_4 representative. Removal of the Ca-oxalate fraction resulted in different mean weight percentages and $\delta^{13}C$ values among the species. Calculated $\delta^{13}C$ values of the Ca-oxalate crystals show depletion from the tissue values in the two C_3 species and enrichment in the C_4 and CAM representatives. The *C. mexicana* samples show the greatest change between the tissue and Ca-oxalates (7.3‰) but the least mean weight percentage (11%), whereas *A. canescens* shows the greatest overall change, with a –2.8‰ isotopic shift and over 48% mean weight percentage. Variability within the samples undergoing each treatment remained relatively unchanged even with increased cellulose purity. This paper provides estimates of isotopic offsets necessary to correct from one treatment to another. Significant differences in $\delta^{13}C$ among different treatments confirm the need to state the tissue fraction analyzed when reporting $\delta^{13}C$ results. *Copyright © 2002 Elsevier Science Ltd*

1. INTRODUCTION

The greatest influence on plant $\delta^{13}C$ results from physiologic mechanisms of carbon acquisition. Models of carbon isotope fixation in C_3 plants show a dynamic process whereby the atmospheric CO_2 availability is controlled through stomatal conductance and enzymatic fractionation during photosynthesis (Farquhar et al., 1982, 1989; Farquhar, 1983). Yet, the acquisition of carbon is not without its own price, because the opening of stomata for CO_2 acquisition results in the conductance of plant water into the atmosphere. Plants characterized by the C_3 photosynthetic pathway successfully balance these needs under a range of environmental conditions, even under extremes in which carbon acquisition must be maximized while minimizing water loss. Over time, additional photosynthesis systems evolved (C_4 and crassulacean acid metabolism [CAM]) that partially decouple the supply and demand function of CO_2 at the site of fixation, allowing for greater plant water retention and competitiveness in extreme environments (Ehleringer et al., 1991). Each of these carbon acquisition systems shows a distinctive range of $\delta^{13}C$ values within the plant tissue. However, variability within the range of values occurs within plants, between cohorts, and among species (Van de Water, 1999) and may result from secondary fractionation during physiologic processes, changes in environmental growth conditions, and/or genotypic control of physiologic set-points (Hall et al., 1993). The role that differential processing to remove various tissue fractions has on interplant variability, before isotopic analysis, is unclear.

Stable isotope variability within plant tissue resulted in the establishment of standard protocols to remove labile tissue fractions. These protocols range from relatively little preparation, such as the use of whole tissue, to complex treatments that remove specific chemical compounds within cellulose. In addition, these procedures eliminate carbon fractions found in noncellulose components such as metabolites (starch, lipids, Ca-oxalates, lignin), carbon compounds (amino acids), and other chemical complexes (reviewed in O'Leary, 1981). For example, significant isotopic changes occur with the removal of hemicellulose and lignin from cellulose during processing in select C_3 and C_4 species (Benner et al., 1987). Similar changes occur with the removal of lipids, which show less ^{13}C than the cellulose fraction in C_3 tomato and cotton plants (Park and Epstein, 1960, 1961) as well as the C_4 plant sorghum (Whelan et al., 1970). Isotopic variability also occurs with individual amino acids (Whelan et al., 1970; Lerman et al., 1974). For example, glutamate and aspartate differ by >2‰ in both cotton and sorghum, whereas malic and citric acid from cotton show a >4‰ difference (Whelan et al., 1970). Similar ranges are reported in the CAM plant *Kalanchoe daigremontiana*, with $\delta^{13}C$ values equal to −12‰ for malate, −15‰ for the starch, and −16 to −18‰ for the remaining insoluble residue (Lerman et al., 1974).

Calcium oxalate is a common metabolite stored as insoluble crystals within plant tissue (Arnott and Pautard, 1970; Arnott, 1973, 1976). Chemical processes are used to remove most unwanted carbon compounds, but the carbon-rich Ca-oxalate is resistant to dissolution (solubility product $\sim 10^{-8.5}$; Graustein

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et al., 1977) and can constitute a significant portion, by weight, of sample tissue. Ca-oxalate crystals result from the physiologic regulation of excess Ca (Franceschi and Horner, 1980; Libert and Franceschi, 1987; Franceschi and Loewus, 1995) and occur as one of two forms: monohydrate ($CaC_2O_4 \cdot H_2O$, whewellite) and dihydrate ($CaC_2O_4 \cdot 2H_2O$, weddellite) (Palache et al., 1951; Leavens, 1968). The specific gravity of the two minerals is 2.21 to 2.23 and 1.94, respectively. The high specific gravity results in Ca-oxalates contributing a significant weight percentage of plant tissue at high concentrations. For example, in select species of cacti, up to 50% of the tissue weight is Ca-oxalate (Rivera and Smith, 1979). Isotopic analysis of cacti spines revealed isotopic enrichment in the Ca-oxalate crystals (−7.3 to −8.7‰) compared to the associated woody fibers (−13.3 to −14.1‰) (Rivera and Smith, 1979). The isotopic values fall within the range that CAM plants exhibit. In contrast, depleted isotopic values are reported from rhubarb leaves, a C_3 angiosperm (Hoefs 1969). However, the isotopic values of –29.3 and –27.8‰ are consistent with the $\delta^{13}C$ range of C_3 angiosperms.

Stable isotope variability in the nonlabile carbon fraction occurs with changes in environmental growth conditions, because of the balance between CO_2 acquisition and plant water conductance. The effect of environmental variability forms the basis for using stable isotopes to measure current and reconstruct past plant ecophysiology. The choice of tissue fraction and processing techniques has become to a great extent standardized within disciplines. For example, most ecological research uses whole plant tissue (e.g., Ehleringer et al., 1991; Donovan and Ehleringer, 1992). In tree-ring research, long-lived individuals are used to reconstruct chronologies of past growth conditions, and therefore, the translocation of noncellulose components is a concern, and the use of cellulose, holocellulose, and α-cellulose is preferred (e.g., Long et al., 1979; Krishnamurthy and Epstein, 1990; Leavitt and Danzer, 1993; Loader et al., 1997; Borella et al., 1998). Many paleoenvironmental studies have standardized to holocellulose to minimize the effect of degradation over long periods of time (Van de Water et al., 1994; Van de Water, 1999; Pedicino et al., in press). However, $\delta^{13}C$ values from both modern and ancient plant samples are also reported using nitrocellulose (e.g., Marino and McElroy, 1991; Marino et al., 1992; Pendall et al., 1999), a processing technique utilized to facilitate deuterium isotope analysis of the same tissue (Epstein et al., 1978).

The use of these different processing techniques has led to difficulty in comparing past results across studies and between disciplines. In this paper, ^{13}C analysis was performed on plant tissue representing the three types of photosynthesis in angiosperms as well as a representative of the C_3 gymnosperms. Tissue was collected from two sites containing five individuals for each species of interest. The samples include the angiosperms *Cowania mexicana* (C_3), *Opuntia* spp. (obligate CAM), and *Atriplex canescens* (C_4), and the gymnosperm *Juniperus osteosperma* (C_3). Study sites were chosen to determine if $\delta^{13}C$ trends associated with processing are consistent between sites with different environmental regimes. This study does not address isotopic trends across environmental gradients but focuses on changes between standard isolated tissue fractions within individual plants at each sample locality. The C_3 plants, *C. mexicana* and *J. osteosperma*, were collected at a similar elevation to minimize the effects of changing CO_2 partial pressures along elevation gradients (Gale, 1972). Both *Opuntia* spp. and *A. canescens*, with their CAM and C_4 physiology, are less affected by changing atmospheric gas concentrations; thus, sites having larger elevation changes were selected.

Tissue from each plant underwent four common extraction treatments as well as a special additional treatment to remove Ca-oxalates. The tissue fractions analyzed include whole tissue, holocellulose, α-cellulose, and nitrocellulose preparations. The procession from whole tissue to nitrocellulose removes increasing amounts of noncellulose tissue fractions. Comparison of variability as more noncellulose components are removed may indicate the differential removal of residual labile and nonlabile carbon components. It is hypothesized that with increased processing, the $\delta^{13}C$ value of the remaining tissue will converge on similar cellulose values and that interplant variability may become reduced as noncellulose fractions are reduced. This study does not propose the standardization of preparation processes but aims at quantifying differences in $\delta^{13}C$ values resulting from commonly used treatment protocols.

2. METHODS

The samples used in this study were selected from plants collected in 1995 along transects in southeastern Utah and south central New Mexico. Each C_3 species was collected at the same elevation but on north- and south-facing slopes to maximize environmental differences while keeping the elevation constant. *J. osteosperma* and *C. mexicana* were collected in southeastern Utah at 1675 m. Carbon acquisition is less constrained by water and CO_2 availability in C_4 and CAM plants, and therefore, plant collections were made at different elevations to maximize environmental conditions. *A. canescens* was collected in southeastern Utah on a north- and south-facing slope at 1225 and 1675 m, respectively. *Opuntia* sp. samples were collected on north-facing slopes in New Mexico at 1675 and 1830 m. The cactus sites were chosen to ensure representation by single species, but because each plant often lacked flowers for identification, they are referred to in this study as *Opuntia* spp. All collections consist of material from five randomly chosen plants at each site. Samples were collected from material growing at each of the four cardinal directions of each plant by removing tissue that received full sun. This resulted in the collection of branches with leaves from *C. mexicana* and *A. canescens* plants, twigs with scales from *J. osteosperma* trees, and pads with spines from the *Opuntia* sp. The collected material was stored in paper bags to allow air drying during transportation and storage in the laboratory before analysis. Equal numbers of leaves, scales, or spines from the four clippings from each plant were pooled together into each isotopic sample. All of the species are evergreen, so each sample reflects an unknown number of growth years.

The isolation of leaf tissue components used standard chemical extraction techniques. All samples were initially ground in a Wiley mill until the material passed through a 20-mesh screen. Ground material was then pouched using commercially available filter material, heat sealed to resemble a tea bag. The least processed tissue type was composed of whole tissue prepared by boiling ground material in hot water for 2 h to remove waxes, carbohydrates, and other complex polysaccharides (Pettersen, 1984). Holocellulose was extracted by placing the pouches in a soxhlet with ethanol-toluene and ethanol washes to remove waxes and oils. Samples were then bleached with acidified Na-chlorite to remove lignin (Green, 1963; Leavitt and Danzer, 1993; Sheu and Chiu, 1995). Residual Ca-oxalate was removed using a procedure that follows Baker (1952). Holocellulose samples were washed in 10% HCl for at least 24 h with two to three changes of the acid solution, then rinsed until neutral. Residual hemicellulose was removed from holocellulose to form α-cellulose by using a strong base, NaOH, followed by glacial acetic acid (Pettersen, 1984). By removing labile hydroxyl groups from α-cellulose, nitrocellulose was isolated

Table 1. Average $\delta^{13}C$ and the range of values (in parentheses) for each site reported for each fraction of cellulose analyzed during the study. The upper and lower values represent the northern and southern aspect in *Juniperus osteosperma*, *Cowania mexicana*, and *Atriplex canescens*. The *Opuntia* sp. samples are from northern aspects, with the upper value representing 1830 m and the lower from 1675 m. Each reported value represents the sum of five plants.

	Juniperus osteosperma	*Cowania mexicana*	*Atriplex canescens*	*Opuntia* sp.
Whole tissue	−24.1 ± 0.9 (1.9)	−24.9 ± 1.1 (2.6)	−14.3 ± 0.1 (0.3)	−11.1 ± 0.5 (1.1)
	−22.1 ± 0.4 (0.8)	−24.6 ± 0.5 (1.1)	−14.8 ± 0.3 (0.8)	−10.5 ± 0.6 (1.5)
Holocellulose	−23.47 ± 0.9 (2.1)	−24.7 ± 0.4 (1.0)	−10.1 ± 0.4 (0.9)	−11.8 ± 0.7 (1.8)
	−21.5 ± 0.4 (0.9)	−23.8 ± 0.5 (1.2)	−11.2 ± 0.9 (1.9)	−11.6 ± 0.7 (1.8)
Treated holocellulose[a]	−23.1 ± 1.1 (2.4)	−24.0 ± 0.9 (2.4)	−12.1 ± 0.5 (1.1)	−12.2 ± 0.4 (1.0)
	−21.2 ± 0.4 (1.1)	−23.0 ± 0.8 (1.8)	−12.0 ± 0.4 (1.2)	−11.7 ± 0.8 (1.6)
α-cellulose	−22.7 ± 0.9 (2.0)	−23.7 ± 0.4 (1.0)	−10.4 ± 1.2 (3.1)	−12.0 ± 0.4 (1.1)
	−20.9 ± 0.5 (1.4)	−22.6 ± 0.5 (1.1)	−11.8 ± 0.2 (0.4)	−11.5 ± 0.7 (1.8)
Nitrocellulose	−22.0 ± 1.1 (2.3)	−23.7 ± 0.4 (0.8)	−13.7 ± 0.7 (1.7)	−13.6 ± 0.9 (2.3)
	−19.9 ± 0.7 (1.6)	−23.1 ± 0.6 (1.3)	−13.3 ± 0.3 (0.7)	−11.9 ± 0.6 (1.4)

[a] Ca-oxalate removed.

(Robertson et al., 1995). The procedure used fuming nitric acid and acetic anhydride (Epstein and Yapp, 1976; Sternberg, 1989).

Each tissue fraction was dried and removed from its pouch, and up to 3 mg were weighed for combustion to CO_2 and H_2O. The material was placed within a Vycor tube with copper oxide as an excess oxygen source during firing. Sample tubes were evacuated and torch sealed, then burned successively at 900° and 650°C for 2 h. Silver foil was added to the nitrocellulose samples along with the Cu-oxide to remove NO_x during firing. Once sealed, the nitrocellulose samples were combusted for 4 h at 800°C (Northfelt et al., 1981). All sample tubes were cracked in vacuo and the CO_2 cryogenically isolated, then measured on a Finnigan MAT Delta-S mass spectrometer. The $\delta^{13}C$ results are reported with respect to the Peedee belemnite standard (Craig, 1957). Each batch of 10 samples was accompanied by a lab standard consisting of a C_3 white spruce cellulose or C_4 sucrose. Results of the C_3 and C_4 standard analysis during this study ($C_3 = -23.8 \pm 0.2$, $n = 11$; $C_4 = -11.0 \pm 0.2$, $n = 21$) are both within 1σ of the long-term value of each material (-23.8 ± 0.2, $n = 164$; $C_4 = -11.0 \pm 0.2$, $n = 107$). To ensure that Ca-oxalates were not present in the white spruce cellulose standard, 5 samples were treated for its removal. Analysis shows values (-23.9 ± 0.1, $n = 5$) falling within 1 SD of the long-term mean standard value. The standard deviations for the C_3 and C_4 standard values fall at or below 0.2‰, the estimated error for laboratory preparation and analysis.

Statistical analysis employed the SPSS software package. Descriptive statistics for all sites suggested normally distributed values. Statistical analysis used one-way analysis of variance comparing the isotopic value of each tissue preparation for the set of plants growing at each site. In addition, differences among treatments by site and species were calculated from pooled values and are reported. By convention, $\delta^{13}C$ differences were calculated by subtracting the isotopic value associated with greatest amount of processing from the lesser processed fraction. For example, α-cellulose values were subtracted from holocellulose values and nitrocellulose from α-cellulose.

Residual Ca-oxalate crystals were first observed in processed holocellulose tissue under microscopic examination. A reanalysis of the same tissue after processing showed its removal. To further identify the observed crystalline material, X-ray powder diffraction analysis was performed on isolated material from a single sample of *A. canescens*. The weight percentage of Ca-oxalate within the holocellulose tissue was determined for each species by weighing samples before and after chemical processing. Samples were weighed on a Mettler (series 6600) electronic balance after equilibration for a 48-h period to a temperature of 24 ± 3°C and a humidity of 50 ± 10%. A standard 100-mg weight measured after each group of five samples returned a consistent value of 100.0 ± 0.1 mg.

3. RESULTS

The carbon isotopic results for the different treatments and plant tissues are shown in Tables 1, 2, and 3. Overall, the $\delta^{13}C$ values of the different tissue types show distinct trends among species categorized by their carbon fixation pathway. The consistency of these trends occurs even though each site was chosen to maximize environmental differences. Intersite variability remains relatively constant across the range of processing techniques (Table 1; Fig. 1). Increased processing resulted in mean $\delta^{13}C$ values becoming less negative in the C_3 plants but more negative in the CAM representative. The C_4 species showed the widest range and greatest variability of values among treatments (Fig. 1). The observation of crystalline material within holocellulose samples led to further characterization and formulation of procedures for its removal. Analysis by X-ray diffraction confirmed the presence of Ca-oxalate (N. Foit, personal communication 2001). The crystalline material isolated from *A. canescens* leaves was identified as nearly pure whewellite, the monohydrate form of Ca-oxalate ($CaC_2O_4 \cdot H_2O$). Microscopic analysis of samples pre- and post treatment confirmed the removal of Ca-oxalate using the added procedure. To ensure the systematic removal of the metabolite from all samples, a similar treatment was undertaken and the holocellulose fraction reanalyzed. Unlike the $\delta^{13}C$ tissue trends, calculated weight percentage and reconstructed $\delta^{13}C$ of the Ca-oxalate combine for a unique result in each species tested (Fig. 2; Table 4).

The C_3 representatives include the angiosperm *C. mexicana* and the gymnosperm *J. osteosperma*. $\delta^{13}C$ values for the *C. mexicana* samples range from –25.6 to –22.2‰. Grouping by treatment and site, the values show the greatest range in whole tissue (2.6‰) and the lowest range in the nitrocellulose (0.8‰) samples, both from the north-facing locality. Mean site values from the south-facing site are relatively stable and show only a 0.7‰ difference (Table 1). Statistical analysis comparing each treatment's $\delta^{13}C$ values at each site showed significant differences at both the north-facing ($F = 3.2$, $df = 4$, $p = 0.04$) and south-facing ($F = 9.0$, $df = 4$, $p < 0.01$) localities. However, the Tukey comparison failed to find specific instances of significance ($p > 0.05$) between treatments on the north-facing slope. $\delta^{13}C$ values at the south-facing locality show that whole tissue is significantly more negative than all other *C. mexicana* fractions. In addition, holocellulose values also differ significantly from α-cellulose (Table 2; Fig. 1). Values for each site also become more similar as the treatments remove noncellu-

Table 2. Statistical analysis of each tissue fraction compared to others at the same site using the Tukey comparison with analysis of variance. Mean $\delta^{13}C$ values and range are given in Table 1.

Statistical comparison	*Juniperus osteosperma*		*Cowania mexicana*		*Opuntia* spp.		*Atriplex canescens*	
	North	South	North	South	1675 m	1800 m	North	South
Whole tissue vs. holocellulose	0.73	0.29	0.99	0.32	0.10*	0.35	<0.01***	<0.01***
Whole tissue vs. treated holocellulose[a]	0.44	0.06*	0.29	<0.01***	0.05**	0.05**	<0.01***	<0.01***
Whole tissue vs. α-cellulose	0.17	0.01**	0.08*	<0.01***	0.11	0.17	<0.01***	<0.01***
Whole tissue vs. nitrocellulose	0.02**	<0.01***	0.10*	<0.01***	0.02**	<0.01***	0.65	<0.01***
Holocellulose vs. treated holocellulose[a]	0.99	0.89	0.55	0.20	1.00	0.83	<0.01***	0.12
Holocellulose vs. α-cellulose	0.79	0.45	0.19	0.03**	1.00	0.99	0.95	0.32
Holocellulose vs. nitrocellulose	0.20	0.01**	0.24	0.23	0.92	<0.01***	<0.01***	<0.01***
Treated holocellulose* vs. α-cellulose	0.97	0.93	0.94	0.83	0.99	0.97	<0.01***	0.98
Treated holocellulose* vs. nitrocellulose	0.42	<0.01***	0.97	1.00	0.99	0.02**	0.01**	<0.01***
α-cellulose vs. nitrocellulose	0.79	0.03**	1.00	0.78	0.89	<0.01***	<0.01***	<0.01***

[a] Ca-oxalate removed.
*$p < 0.10$. **$p < 0.05$. ***$p < 0.01$.

lose components. For example, the difference between whole tissue and holocellulose is 0.2 and 0.8‰, but between α-cellulose and nitrocellulose, it is 0.0 and 0.5‰ for north- and south-facing sites, respectively. Yet, the range of $\delta^{13}C$ values among individual *C. mexicana* plants for each of the treatments does not change systematically. Calculation of the weight percentage of Ca-oxalate within the leaf tissue showed values of ~10% (Table 4; Fig. 2). Calculation of the mean Ca-oxalate $\delta^{13}C$ value is –30.8‰, the largest difference between metabolite and the surrounding tissue of any of the species tested. Yet, the small weight percentage resulted in only minor mean differences between the $\delta^{13}C$ of the treated (–23.5‰) and untreated (–24.3‰) holocellulose samples, a change of <1.0‰.

A trend of increasing $\delta^{13}C$ during processing also occurs in the C_3 gymnosperm *J. osteosperma*. $\delta^{13}C$ analysis shows values ranging between –25.1 and –19.0‰. Mean treatment values for each site show enrichment in ^{13}C compared to the C_3 angiosperm *C. mexicana*. *J. osteosperma* values grouped by treatment and site show the greatest range (2.4‰) in treated holocellulose from the north-facing slope and the lowest range (0.8‰) in whole tissue growing on the south-facing locality (Table 1). The ranges of values are relatively stable at both localities, differing by only 0.5‰ on the north-facing site and 0.8‰ on the south-facing slope. Statistical analysis comparing the mean $\delta^{13}C$ treatment values at each site showed significant differences at the north-facing ($F = 3.4$, $df = 4$, $p = 0.03$) and

Table 3. Each value represents the average difference among plant treatments for each of the species reported in this study. Values are calculated by averaging isotopic differences between fraction of the same plant. All of the plants from each species are averaged into each specific treatment value. The sample having the greatest amount of material removed was consistently subtracted from the sample with the least.

$\Delta\delta^{13}C$	*Juniperus osteosperma*	*Cowania mexicana*	*Atriplex canescens*	*Opuntia* spp.
Whole tissue–holocellulose	−0.7 ± 0.8	−0.5 ± 0.8	−3.9 ± 0.8	0.9 ± 0.4
Whole tissue–treated holocellulose[a]	−1.0 ± 0.8	−1.2 ± 0.6	−2.5 ± 0.6	1.2 ± 0.4
Whole tissue–α-cellulose	−1.3 ± 0.9	−1.6 ± 0.6	−3.4 ± 0.9	1.0 ± 0.6
Whole tissue–nitrocellulose	−2.1 ± 1.0	−1.0 ± 1.2	−1.1 ± 0.7	2.0 ± 0.9
Holocellulose–treated holocellulose[a]	−0.3 ± 0.2	−0.8 ± 0.5	1.4 ± 0.9	0.3 ± 0.4
Holocellulose–α-cellulose	−0.6 ± 0.2	−1.1 ± 0.4	0.5 ± 1.1	0.1 ± 0.6
Holocellulose–nitrocellulose	−1.5 ± 0.4	−0.9 ± 0.4	2.8 ± 1.2	1.1 ± 1.1
Treated holocellulose[a]–α-cellulose	−0.3 ± 0.4	−0.4 ± 0.4	−0.9 ± 1.2	−0.2 ± 0.7
Treated holocellulose[a]–nitrocellulose	−1.2 ± 0.3	−0.2 ± 0.5	1.5 ± 0.8	0.8 ± 1.1
α-cellulose–nitrocellulose	−0.8 ± 0.4	0.2 ± 0.2	2.4 ± 1.3	1.0 ± 0.9

[a] Ca-oxalate removed.

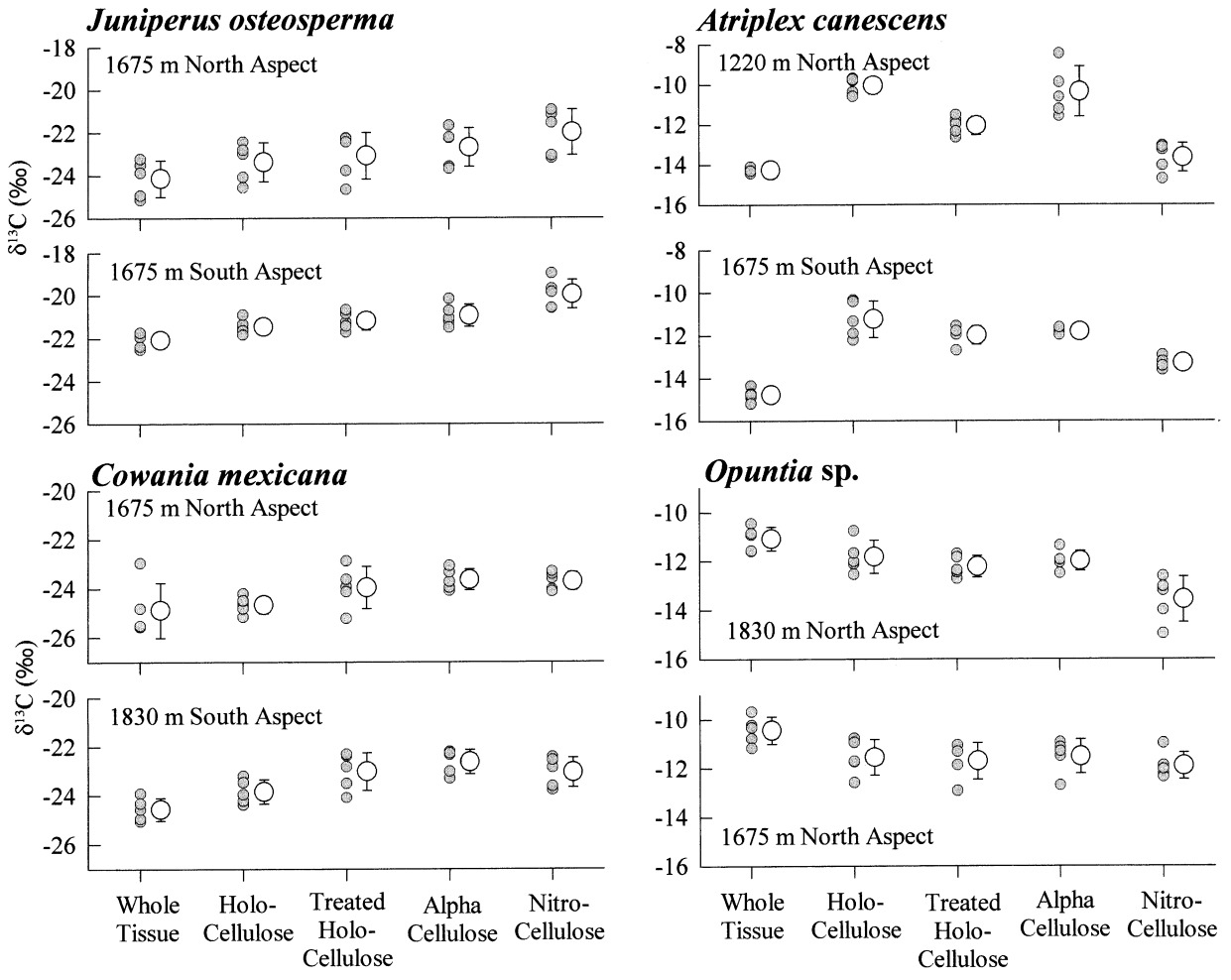

Fig. 1. Reported δ^{13}C values for the different tissue treatments used in this study. The small gray circles represent individual values, and the larger circles represent the means with error bars of 2σ.

south-facing ($F = 13.1$, $df = 4$, $p < 0.01$) localities. The Tukey comparison for the north-facing slope shows a single comparison with significantly different values, whole tissue vs. nitrocellulose (Table 2). On the south-facing slope, whole tissue is significantly depleted in ^{13}C compared to α-cellulose. In addition, the nitrocellulose fraction is significantly enriched in ^{13}C compared to all treatments (Table 2). Unlike *C. mexicana*, there is no apparent trend toward similar values with increased processing in *J. osteosperma*. However, for each treatment, the range of values does remain relatively stable. This holds comparing either site values or all values grouped by species (Table 3). The calculated weight percentage of Ca-oxalate in *J. osteosperma* shows the greatest range of values in this study (21 to 68%), with a mean value of 40% (Table 4; Fig. 2). Calculation of the mean δ^{13}C Ca-oxalate value is –22.8‰, a value close to the surrounding tissue (Table 4). This similarity results in a mean difference between the treated (–22.1‰) and untreated samples (–22.4‰) of only 0.3‰.

Opuntia spp. spine tissue shows a consistent trend of decreasing ^{13}C with sample treatment. The isotopic shift is opposite to the C_3 plants *J. osteosperma* and *C. mexicana*. The range of δ^{13}C values in *Opuntia* spp. is from –9.7 to –15.0‰. Grouping by treatment and site, the greatest range of values occurs in the nitrocellulose samples from the 1830-m site. All of the other values fall below 2.0‰ with the smallest range occurring in treated holocellulose, also at the 1830-m locality. Values at the 1675-m site are relatively stable, with the difference between ranges falling below 0.5‰ (Table 1; Fig. 1). Statistical analysis of the mean values for each treatment shows statistically significant differences for sites at 1830 m ($F = 10.8$, $df = 4$, $p < 0.00$) and 1675 m ($F = 3.7$, $df = 4$, $p = 0.02$), respectively. The Tukey comparison for the 1830-m locality shows the nitrocellulose sample differs significantly from all the other treatments. At the 1675-m locality, significant differences occurred between whole tissue and treated holocellulose as well as nitrocellulose (Table 2; Fig. 1). Increased treatment and greater removal of noncellulose components resulted in more negative values in all cases except the comparison between treated holocellulose and α-cellulose (Table 3). However, there appears to be no trend in the difference between mean pretreatment values or the range of individual *Opuntia* spp. values (Table 1; Fig. 1). Calculated Ca-oxalate weight percentage shows the second lowest range of values, 13 to 23%, with a mean of 16% (Table 4; Fig. 2). The calculated

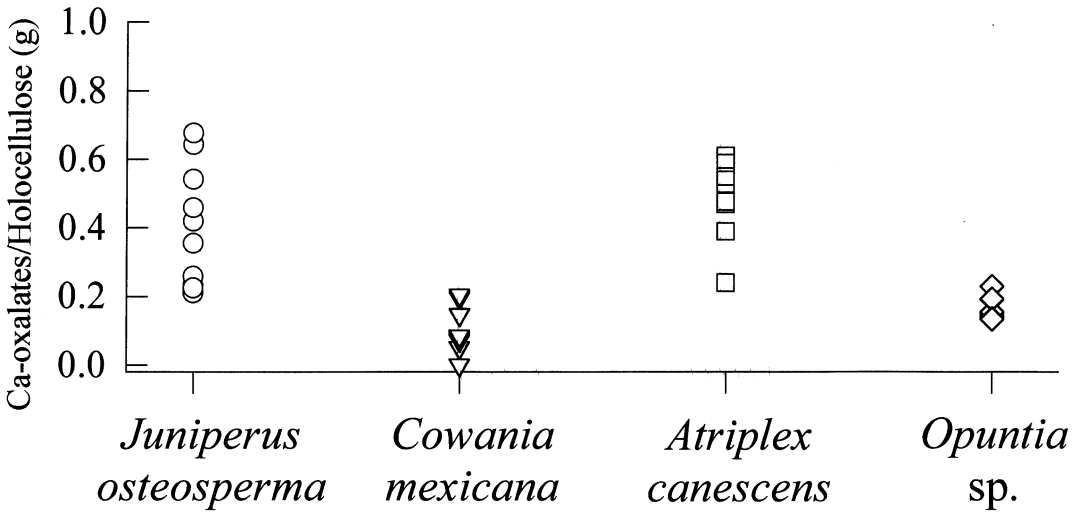

Fig. 2. Weight percentage Ca-oxalate for each species in this study. Comparison with Table 4 reveals species-specific changes in the weight percentage and $\delta^{13}C$ value of the Ca-oxalate. For example, similar values in the two C_3 species result from different characteristics. The *Juniperus osteosperma* samples have high weight percentages but $\delta^{13}C$ values close to those of the holocellulose, whereas *Cowania mexicana* has only minor percentages but significantly different $\delta^{13}C$ values.

Ca-oxalate $\delta^{13}C$ value is –10.2‰, with the surrounding tissue at –12.0‰, a difference of 1.8‰. The reduced weight percentage resulted in a $\delta^{13}C$ change between the treated (–11.7‰) and untreated (–12.0‰) samples of only 0.3‰ (Table 4).

The C4 plant *A. canescens* shows the greatest range of mean values among the species tested at each site (Table 1; Fig. 1) as well as when grouped by treatment (Table 3). The $\delta^{13}C$ values for all samples varied from –15.2 to –8.5‰, a range of 6.7‰. Grouped into each treatment and by site, the greatest range occurs in the α-cellulose and the lowest range in the whole tissue samples from the north-facing locality (Table 1; Fig. 1). Mean site values at the south-facing site differ by <2.0‰. Statistical analysis comparing treatments shows significant differences at both the north-facing ($F = 36.2$, $df = 4$, $p < 0.01$) and south-facing ($F = 45.7$, $df = 4$, $p < 0.01$) localities (Table 2). Trends among treatment techniques are less clear cut than in the other species (Tables 1 and 2; Fig. 1). For example, whole tissue is depleted in $\delta^{13}C$, but the holocellulose samples show the greatest enrichment. However, once treated, the holocellulose samples become less enriched in ^{13}C, but values remain less negative than the whole tissue. At the north-facing site, the α-cellulose samples are significantly different than their treated holocellulose counterpart, but this difference does not occur at the south-facing locality. Similarly, greater differences occur between the α-cellulose and nitrocellulose samples on the north- compared to south-facing sites. Calculation of the Ca-oxalate weight percentages shows the highest mean value (48%) in the study (Table 4; Fig. 2). Calculation of the mean $\delta^{13}C$ Ca-oxalate values is –9.2‰, the most enriched value of any species tested. This combined with the significant weight percentage of Ca-oxalate present results in mean values between the holocellulose and treated holocellulose fractions being >1.4‰ (Table 4; Fig. 2). The $\delta^{13}C$ of Ca-oxalate differs by –2.8‰ from the holocellulose.

4. DISCUSSION

Analysis of the different tissue fractions resulting from standardized processing shows broad-based trends. Each of the treatments resulted in $\delta^{13}C$ values reflecting the range peculiar to the specific type of photosynthetic physiology. However, the trend from treatment to treatment changed with the increased removal of labile carbon compounds; the C_3 species show isotopic enrichment, the CAM species became depleted, and mixed results occurred in the C_4 plants. It was expected that with increased processing, isotopic differences would converge toward a systematic value associated with the pure cellulose fraction. Only in the *C. mexicana* does this trend occur. Similarly, it was hypothesized that if differential amounts of noncellulose components were contributing to sample variability,

Table 4. Reconstructed $\delta^{13}C$ Ca-oxalate values for each of the four species. Values were calculated using the pre- and postweight of each sample along with the $\delta^{13}C$ of the treated and untreated holocellulose fractions.

Species	Weight percentage (%)	δ^{13} holocellulose (‰)	$\delta^{13}C$ treated holocellulose (‰)	$\delta^{13}C$ Ca-oxalate (‰)	Δ (treated holocellulose–Ca-oxalate) (‰)
Juniperus osteosperma	40.2	−22.4	−22.1	−22.8	0.70
Cowania mexicana	10.5	−24.3	−23.5	−30.8	7.30
Opuntia spp.	16.1	−11.7	−12.0	−10.2	−1.80
Atriplex canescens	48.4	−10.6	−12.0	−9.2	−2.80

as processing progressed, the range of values would systematically decrease. The range of values for each treatment shows few if any trends across the data set. It is therefore suggested that the inherent intra- and intersite variability results from factors not associated with the removal of labile carbon components. Recognition of residual Ca-oxalate crystals within the holocellulose samples resulted in an additional treatment being added and results reported. Calculated weight percentages and reconstructed difference between Ca-oxalate $\delta^{13}C$ and tissue values are variable among the species and inconsistent with the divisions by photosynthetic pathway. However, trends exist with the Ca-oxalate from the C_3 plants consistently depleted in $\delta^{13}C$ and the CAM and C_4 species enriched, as reported previously (Hoefs, 1969; Rivera and Smith, 1979).

The formation of Ca-oxalate in plants depends upon the availability of Ca and evapotranspiration demands (Frank, 1972; Franceschi and Horner, 1979; Kirkby and Pilbeam, 1984; Franceschi and Loewus, 1995). The greatest weight percentage of Ca-oxalate was found in the *J. osteosperma* samples. However, the greatest difference between the surrounding holocellulose and the metabolite occur in *C. mexicana* and *A. canescens*, both of which grow at low elevations and in extreme environments. In fact, even greater differences between Ca-oxalate and holocellulose are reported for *A. canescens* and *A. confertifolia* samples from the same area (Van de Water, 1999). If environmental extremes are driving the isotopic differences, it suggests that *Opuntia* spp. spines coming from extreme growth conditions should show similar differences. Significant Ca-oxalate concentrations are reported from cactus growing in west Texas (Rivera and Smith, 1979). The older cortex of *Echinomastus* and *Echinocactus* reportedly contains up to 50% dry weight Ca-oxalate, and $\delta^{13}C$ values for Ca-oxalates in the pads show ^{13}C enrichment compared to the surrounding tissue. Cactus spine values in this study are consistent with previous findings of isotopic enrichment. However, the weight percentages are greatly reduced and may result from the lack of vascular tissue within the organ (Nobel, 1988). These results suggest that the failure to remove Ca-oxalates within sample tissue may significantly alter study results. In addition, broad-based surveys of Ca-oxalate occurrence and isotopic signatures need to be undertaken to investigate environmental conditions governing their formation within plant tissue.

Chemical processing of whole leaf tissue to holocellulose aims to remove all but the cellulose fraction, including metabolic and nonmetabolic compounds (Pettersen, 1984). The material removed includes noncellulose compounds that show a wide range of $\delta^{13}C$ values (O'Leary, 1981). This suggests that the overall contribution of ^{13}C from these compounds to the whole tissue isotope value is minimal. This could occur if (a) the mean value of the combined metabolic and nonmetabolic fraction is close to the cellulose value, (b) contributions from these fraction make up a small percentage of the total tissue measured for the $\delta^{13}C$ value, or (c) these compounds are easily degraded and/or removed during the limited preparation that whole tissue samples undergo. Comparisons of whole tissue and holocellulose values show $<1.0‰$ difference in three of the species tested, but *A. canescens* differs by 3.9‰ (Table 2). These results may be misleading if residual Ca-oxalate contributes a significant percentage of each isotopic value. In fact, comparison of whole tissue with holocellulose treated to remove Ca-oxalate shows an increased difference in the C_3 and CAM representatives and a decrease with *A. canescens*. The difference between the treated holocellulose and whole tissue values become $>1.0‰$.

Further processing of each sample removes the hemicellulose fraction, resulting in the isolation of α-cellulose. Hemicellulose is a mixture of polysaccharides with lower molecular weights than α-cellulose (Pettersen, 1984). The C_3 α-cellulose samples show enriched ^{13}C values compared to their holocellulose counterparts, whereas the *A. canescens* and *Opuntia* spp. samples become depleted in ^{13}C (Fig. 2). The depletion in the C_4 representative is reversed if the treated holocellulose is used, which again points to the significance of residual Ca-oxalates within the holocellulose preparation. The changes associated with processing from whole tissue to α-cellulose are consistent with the work of Benner et al. (1987). Their study reports ^{13}C enrichment in a hemicellulose and α-cellulose fraction compared to whole tissue in two C_3 and C_4 species. The C_3 and C_4 values shift by -0.9 and $-1.23‰$, respectively, which is less than the -1.6 and $-3.4‰$ shift reported in this study (Table 3). This occurs with the removal of an uncharacterized ^{13}C-depleted fraction (lipids, carbohydrates, and amino acids) and ^{13}C-depleted lignin. Both results are consistent with trends in this study, in which the treated holocellulose values are used. Processing from α-cellulose to nitrocellulose removes labile protons in exchangeable hydroxyl groups during the conversion. The results continue the trend in the C_3 species, with enriched values suggesting the additional removal of ^{13}C-depleted carbon fractions. These enriched values are consistent with the results of Robertson et al. (1995) from analysis of C_3 wood cellulose from *Quercus petraea*. However, the shift in isotopic values between α-cellulose and nitrocellulose is reported as -0.2 to $-0.3‰$, whereas in this study, they shift by $-0.8‰$ (Table 3). In the C_4 and CAM representatives, $\delta^{13}C$ values become depleted, suggesting the removal of an enriched fraction. In both cases, it is unknown why the removal of hydroxyl groups leads to shifts in the $\delta^{13}C$ value of the remaining cellulose fraction. The removal of residual polysaccharides remaining from incomplete processing in a previous step must be considered.

Throughout the analysis of different tissue fractions, the variability associated with each site failed to show a reduction as hypothesized. This indicates that the variability associated with individual plants is determined by factors other than differential removal of noncellulose components in the samples during pretreatment before analysis. If true, then interstudy comparisons are possible by adding predetermined constants to analysis results for different tissue fractions (Table 3). However, caution is warranted when comparing the limited results that are available. For example, whole tissue $\delta^{13}C$ values for gymnosperms are estimated by subtracting 1.5‰ from measured holocellulose values (Leavitt and Long, 1982; Van de Water et al., 1994; Pendall et al., 1999). If the *J. osteosperma* values from this study reflect gymnosperms in general, then these corrections were overstated. In addition, tissue fraction differences in both Benner et al. (1987) and Robertson et al. (1995) are less than the results from the corresponding photosynthetic type reported in this study. Additional analysis needs to be undertaken to assess these responses.

5. CONCLUSIONS

The isotopic analysis of tissue fractions from leaf, twig, and spine samples revealed significant differences between representatives of C_3, C_4, and CAM physiologies. With increased processing, more ^{13}C was found in the C_3 samples, whereas more negative isotopic values occurred in CAM representatives. The greatest range and the most variable change between treatments occurred in *A. canescens*. During the study, residual Ca-oxalate was discovered in holocellulose samples. The weight percentage and isotopic value of the Ca-oxalate varies by species, although the $\delta^{13}C$ values remain in the range associated with each carbon acquisition pathway. A hypothesized reduction in the range of values with increased processing, intersite variability, is unsupported in the findings. Similarly, a reduction in the difference between sample values as the purity of cellulose increased is not consistently observed. The effect of different treatment techniques on the $\delta^{13}C$ of plant tissue points to the necessity of stating the procedures and correction factors used when reporting study results.

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