1 Microbial alteration of the hydrogen and carbon isotopic composition of *n*-alkanes in sediments

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Abstract

Long-carbon chain length n -alkanes ($n\text{-C}_{27}\text{--}n\text{-C}_{33}$) are commonly produced by terrestrial plants and preserved in sedimentary archives. The hydrogen (δD) and carbon ($\delta^{13}\text{C}$) isotope compositions of these compounds provide a record of paleohydrologic and paleoecologic conditions at the time of formation. At present, the role bacteria play in altering the stable isotope composition of biomarker compounds during storage of sediments is unclear. We collected four sediment samples at precise depth intervals from an archaeological site and stored these samples at room temperature for three years. We collected sediment from identical locations (precise to the cm level) three years later and froze these immediately. All stored samples showed high amounts of short- and medium-chain length n -alkanes ($n\text{-C}_{18}\text{--}n\text{-C}_{26}$), which were absent from samples that were immediately frozen. Measured δD values of $n\text{-C}_{29}$ and $n\text{-C}_{31}$ n -alkanes increased in stored samples by up to 25‰. $\delta^{13}\text{C}$ values in stored samples are more negative than non-stored samples by up to 4.2‰. DNA analysis of the soil microorganisms showed bacteria containing genes coding for n -alkane degrading enzymes proliferated in samples during their three year storage. In particular, *Rhodococcus* and *Aeromicrobium*, genera which contain multiple coding regions for n -alkane degrading enzymes *CYP153* and *AlkB*, increased by an order of magnitude (from 0.7% to 7.5% of bacteria present). These data suggest that bacteria may play a larger role than previously expected in altering measured n -alkane stable isotope values during storage and that analyses conducted on stored samples may lead to erroneous results.

1. Introduction

Plants produce a wide range of compounds as part of their epicuticular wax that protect the plant from water loss and UV damage (Sutter and Langhans, 1982; Long et al., 2003; Jetter et al., 2006). Among these compounds are *n*-alkanes that show a strong odd-over-even predominance of long-chains (*n*-C₂₅–*n*-C₃₅) in terrestrial plants (Eglinton and Hamilton, 1967). Carbon isotope ($\delta^{13}\text{C}$) values in *n*-alkanes generally reflect the photosynthetic pathway of plants and responses to external environmental conditions (Collister et al., 1994; Castaneda et al., 2009; Diefendorf et al., 2011, 2015) though the magnitude of isotope fractionation associated with lipid biosynthesis is not fully understood (Diefendorf et al., 2011; Dungait et al., 2011; Eley et al., 2016). $\delta^{13}\text{C}$ values of *n*-alkanes are commonly used to measure changes in vegetation, carbon cycle and moisture availability (e.g., Beerling and Osborne, 2006; Smith et al., 2007; Tipple and Pagani, 2007; Handley et al., 2008; Whiteside et al., 2010; French et al., 2014; Uno et al., 2016a, 2016b; Diefendorf and Freimuth, 2016). The hydrogen isotope (δD) composition of *n*-alkanes records the δD value of environmental water during the formation of the leaf waxes, which in terrestrial plants predominantly reflects meteoric water δD values (Sachse et al., 2012). This is further modified by processes such as temperature, aridity, plant functional type, and timing of wax formation (Sessions et al., 1999; Chikaraishi and Naraoka, 2003; Chikaraishi et al., 2004; Sachse et al., 2006; Hou et al., 2007a, 2007b; McInerney et al., 2011; Yang et al., 2011; Kahmen et al., 2013; Tipple et al., 2013, 2015; Eley et al., 2014; Gao et al., 2014; Oakes and Hren, 2016; Gamarra et al., 2016). Since meteoric water δD values vary as a function of temperature, altitude, amount of precipitation and cloud history (Dansgaard, 1964; Gat, 1996), *n*-alkanes record information that is useful for paleoenvironmental studies. Application of δD values in *n*-alkanes include studies on paleoelevation, hydrological and ecosystem changes (e.g., Schefuß et

al., 2005; Pagani et al., 2006; Polissar et al., 2009; Hren et al., 2010; Magill et al., 2013) and may be used to examine changes to plant function over deep geologic time (e.g., Wilson et al., 2015).

Use of molecular biomarker δD and $\delta^{13}C$ for paleoenvironmental reconstruction requires preservation of primary isotopic compositions over the timescale of study. The strength of the C-H bond is shown to minimize significant change in the C or H isotopic composition of saturated *n*-alkanes at temperatures below 150 °C (Schimmelmann et al., 1999). Thus, during shallow sedimentary burial, it is unlikely that *n*-alkanes will exchange H or C isotopes. Recent data does suggest there may be isotope effects during the degradation of the leaf (Zech et al., 2011). The process of soil formation or burial in marine sediments could also impart an isotopic shift. A relatively unknown source of isotopic alteration is during sample storage after collection. Typically, this is not assumed to impart any potential bias of saturated hydrocarbon isotopes.

Bacterial degradation of *n*-alkanes is common in aerobic environments and has been identified in a variety of natural environmental settings (Whyte et al., 2002; Quatrini et al., 2008; Shibata and Robert, 2009; Kuhn et al., 2009; Powell et al., 2010; Panicker et al., 2010; Jurelevicius et al., 2012; De Pasquale et al., 2012; Hassanshahian et al., 2013, 2014; Bayat et al., 2015). Dellagnezze et al. (2014) found that up to 99% of *n*-alkanes ranging in length from *n*-C₁₄–*n*-C₃₃ were degraded by bacteria under controlled conditions in as short as 21 days (Dellagnezze et al., 2014). Microbial alteration of *n*-alkane abundances has been noted during long term storage of soil samples (Grimalt et al., 1988). However, the isotope effects associated with this process are unknown.

We collected sediment from precise sample depths at an archaeological excavation site in the Republic of Armenia. Due to the archaeological nature of the sample area, sediments were collected from a narrow clean-cut face within a 2 m wide excavation at 2 cm intervals, with

precision equivalent to a terrestrial sediment core (equivalent to splits of a core sample). One set of samples (Set A) was collected in 2014 and lyophilized to be sure of no microbial activity, and another (Set B) was collected in 2011 and allowed to sit for three years in uncontrolled storage conditions at the University of Connecticut (ambient room temperature). Following this, *n*-alkanes were lyophilized, extracted, quantified, and the compound-specific δD and $\delta^{13}C$ values were measured. In addition, we analyzed the genomes of sedimentary bacteria and fungi to determine changes in the microbial assemblages present in the samples during storage.

2. Material and methods

2.1 Sample Collection

We collected sediment samples (~25 g) in the summer of 2011 from the archaeological site of Lusakert Cave, Armenia, part of the Hrazdan Gorge Paleolithic Project (Adler et al., 2012; Gasparian et al., 2014). These samples were stored in sealed plastic bags at room temperature and three years (2014) later extracted to test *n*-alkane preservation at the site. Samples were not opened after collection until 2014. After the identification of *n*-alkanes preserved in the 2011 samples, we recollected samples (~100 g) from identical positions along the re-cleaned stratigraphic section (sedimentary core equivalent) in July 2014. We stored this second set of samples frozen, and they were lyophilized three days after collection prior to lipid extraction.

*2.2 Extraction, Separation and Quantification of *n*-alkanes*

For all samples, organics were extracted from the sediment via Soxhlet extraction in 2:1 dichloromethane:methanol for 48 hours. Following lipid extraction, *n*-alkanes were separated by silica gel column chromatography and quantified on a Thermo-Scientific Trace GC Ultra

(Manufacturer) fitted with a split–splitless (SSL) injector and flame ionization detector (FID) using a BP-5 column (30 m × 0.25 mm i.d., 0.25 µm film thickness) with He as the carrier (1.5 ml/min). Odd over even predominance (OEP) (Eq. 1) and average chain length (ACL) (Eq. 2) were used to evaluate distributions of *n*-alkanes (Oakes and Hren, 2016).

$$\text{OEP} = \frac{C_{25}+C_{27}+C_{29}+C_{31}+C_{33}}{C_{24}+C_{26}+C_{28}+C_{30}+C_{32}} \quad \text{Eq. 1}$$

$$\text{ACL} = \frac{23 \times C_{23} + 25 \times C_{25} + 27 \times C_{27} + 29 \times C_{29} + 31 \times C_{31} + 33 \times C_{33}}{C_{23} + C_{25} + C_{27} + C_{29} + C_{31} + C_{33}} \quad \text{Eq. 2}$$

2.3 Stable Isotope Analysis

The δD and δ¹³C values of individual *n*-alkanes were measured with a GC-Isolink coupled to a MAT 253 IRMS (Thermo Scientific) with a BP-5 column (30 m × 0.25 mm i.d., 0.25 µm film thickness). Oven temperature was set at 50 °C for 1 min, ramped to 180 °C at 12 °C/min, then ramped to 320 °C at 6 °C/min and held for 4 min. Internal isotopic standards (Mix A from A. Schimmelman) were analyzed every four samples across a range of concentrations to correct for size and scale effects. Standard deviations were 0.5‰ for δ¹³C and 4‰ for δD. Stable isotopic composition is expressed in standard delta notation relative to VPDB and VSMOW.

2.4 DNA Analysis

2.4.1 DNA extraction, PCR amplification, and sequencing of taxonomic marker

DNA was extracted from 0.25g of soil sample using the MoBioPowerMag Soil 96 well kit (MoBio Laboratories, Inc) according to the manufacturer’s protocol for the Eppendorf epMotion liquid handling robot. DNA extracts were quantified using the Quant-iTPico Green kit (invitrogen, ThermoFisher Scientific). Partial bacterial 16S rRNA genes (V4) were amplified

using 30 ng extracted DNA as template. The V4 region was amplified using 515F and 806R with Illumina adapters and dual indices (8 basepair overlap on 3' (Caporaso 2012), and 8 basepair on the 5' (Kozich 2013)). Samples were amplified in triplicate using Accuprime Supermix (Invitrogen) with the addition of 10 µg BSA (New England BioLabs). The PCR reaction was incubated at 95 °C for 3.5 minutes, the 30 cycles of 30 s at 95.0 °C, 30 s at 50.0 °C and 90 s at 72.0 °C, followed by final extension at 72.0 °C for 10 minutes. PCR products were pooled for quantification and visualization using the QIAxcel DNA Fast Analysis (Qiagen). PCR products were normalized based on the concentration of DNA from 250-400 bp then pooled using the QIAgility liquid handling robot. The pooled PCR products were cleaned using the Gene Read Size Selection kit (Qiagen) according to the manufacturer's protocol. The cleaned pool was sequenced on the MiSeq using v2 2x250 base pair kit (Illumina, Inc).

2.4.2 Sequence data processing and statistical analyses

Sequences were processed in Mothur v. 1.36.1 following the MiSeq SOP (Kozich et al., 2013). After demultiplexing and quality checking steps the sequences were clustered at 97% similarity. Alpha and beta diversity statistics were calculated by subsampling to 10,000 reads per sample. NMS and Permanova were run using the vegan package (Oksanen 2015) in R 3.2.0. A subsampled species matrix was used for indicator species analysis (DeCaceres 2009) and generating a heatmap.

3. Results

3.1 n-alkane abundances and distributions

Sediment stored for three years prior to extraction (Set B) show significantly more short- and medium-chain *n*-alkanes (between *n*-C₁₈ and *n*-C₂₆) than samples that were immediately dried and frozen (Set A) (Fig 1, Table S1). These short- and medium-chain length *n*-alkanes lack

an odd-over-even predominance (Table 1, OEP 2.2–3.5), and are typical of microbial metabolism (Johnson and Calder, 1973). Samples in Set A lack short- and medium-chain length *n*-alkanes, and are comprised almost entirely of *n*-alkanes between *n*-C₂₅ and *n*-C₃₃ characterized by a strong odd-over-even predominance (OEP 6.7–11.7), typical of higher terrestrial plants (Eglinton and Hamilton, 1967). The ACL of Set A (29.2–29.5) is systematically lower than ACL in Set B (27.1–28.4) (Table 1). The concentration of long-chain length *n*-alkanes is lower in samples in Set B (Table S1) by 16–62%, showing removal of *n*-alkanes and not addition through mixture from other sources after sample (e.g., sampling bags).

3.2 *n*-alkane δD and $\delta^{13}C$

The $\delta D_{n\text{-alkanes}}$ in Set A range from –176‰ to –191‰ for *n*-C₂₉ and –182‰ to –200‰ for *n*-C₃₁ (Table 1). Samples in Set B are consistently more enriched in deuterium, with *n*-C₂₉ ranging from –166‰ to –172‰ and *n*-C₃₁ ranging from –166‰ to –185‰. The difference in δD of *n*-C₂₉ and *n*-C₃₁ between Set A and Set B was significant ($p < 0.01$, student's 2-tailed t-test assuming equal variance). The $\delta^{13}C$ of the long-chain *n*-alkanes in Set A range from –28.7‰ to –34.8‰ for *n*-C₂₇; –30.7‰ to –36.1‰ for *n*-C₂₉; –32.7‰ to –36.1‰ for *n*-C₃₁; and –32.8‰ to –36.3‰ for *n*-C₃₃ (Table 1). Three of four samples in Set B are more negative in long chain *n*-alkanes than corresponding samples in Set A, with the exception of Sample 1, which is more positive in three of the four long-chain *n*-alkanes ($\Delta\delta^{13}C$: *n*-C₂₇ = 0.6‰, *n*-C₂₉ = 0.2‰, *n*-C₃₃ = 0.2‰). The largest shift we observe for each long-chain *n*-alkane is –4.4‰ for *n*-C₂₇ (Sample 3), –4.2‰ for *n*-C₂₉ (Sample 2), –2.9‰ for *n*-C₃₁ (Sample 2), and –3.3‰ for *n*-C₃₃ (Sample 2). The $\delta^{13}C$ values of the two sample sets are significantly different ($p < 0.05$, student's 2-tailed t-test assuming equal variance).

3.3 DNA Results

We analyzed the DNA profile within the eight sediment samples to quantify changes in the microbial assemblages within stored and fresh samples. No measurable fungal DNA was identified in the sediments. Four samples, two each from Set A and Set B, yielded more than 10,000 microbial DNA sequences and were included in the analysis. In order to analyze changes in microbial communities related to *n*-alkane degradation, we compared the presence of bacterial genera which are shown to contain genes coding for two *n*-alkane degrading enzymes, *alkB* and *CYP153* (Nie et al., 2014). These are broken down further into three groups (with possible overlap between the groups): genera containing both *alkB* and *CYP153*, genera with multiple copies of *alkB*, and genera with multiple copies of *CYP153*. Out of 16 previously identified bacterial genera with both *alkB* and *CYP153* coding regions, 12 were identified in our samples (Table S2). In Set A, these represent 2695 of 57633 total sequences present (4.67%). In Set B, they represent 12168 of 115172 total sequences present (10.57%). Thirteen genera containing multiple copies of *alkB* were present in our samples, out of 26 previously identified genera (Table S3). In Set A these represent 2237 of 57633 total sequences present (3.88%). In Set B, they represented 14518 of 115172 total sequences present (12.61%). Nine out of 13 previously identified genera with multiple copies of *CYP153* were identified in our samples (Table S4). In Set A these account for 456 of 57633 total sequences present (0.79%). In Set B, they are 8661 of 115172 total sequences present (7.52%).

4. Discussion

We observe five major changes to our samples during storage: (1) an increase in short- and medium-chain length *n*-alkanes, (2) a decrease in the abundance of long-chain length *n*-alkanes, (3) more positive δD values for long-chain length *n*-alkanes, (4) more negative $\delta^{13}C$ values in long-chain length *n*-alkanes, and (5) an increase in the presence of bacterial genera containing coding regions for enzymes used in *n*-alkane degradation. Together, these data are consistent with microbial breakdown *n*-alkanes during storage. The elevated δD values in the samples during storage are correlated strongly with the change in OEP of the samples in both *n*-C₂₉ ($p = 0.004$, one way ANOVA) and *n*-C₃₁ ($p = 0.043$, one way ANOVA) (Fig 2a,b). This is not the case with $\delta^{13}C$ values however, and both *n*-C₂₉ ($p = 0.359$, one way ANOVA) and *n*-C₃₁ ($p = 0.155$, one way ANOVA) *n*-alkanes show no significant correlation with the magnitude of change of OEP (Fig 2c,d). This may be due to *n*-alkane catabolism by bacteria, through oxidation of a hydrogen to convert the molecules to *n*-alkanoic acids (Ji et al., 2013).

All three categories of bacteria with coding regions for *n*-alkane degrading enzymes are present in higher percentages in Set B than Set A (Fig 3). This indicates that during storage of the samples, bacteria with the ability to degrade *n*-alkanes proliferated in the sediments. The changes in the microbial community are driven by two genera in particular, *Rhodococcus* and *Aeromicrobium*, both of which have been identified as containing multiple coding regions of both *alkB* and *CYP153* alkane monooxygenases. Given that these samples came from Pleistocene deposits, at least 45,000 years old, it is most likely that these bacteria were either a) introduced during sample collection or b) present in the soil but multiplied due to aeration of the sediment and exposure to atmospheric moisture during sample collection. Microbial activity is low in buried sediments due to low oxygen and nutrient availability (Leahy and Colwell, 1990). Aeration of sediments enhances microbial activity and hydrocarbon degradation of petroleum

contaminated soils (Ueno et al., 2006; Vieira et al., 2009; Jain et al., 2011; Ros et al., 2014; Smith et al., 2015). Therefore, aeration of sediment is an unintended consequence of sampling, and leads to enhanced activity of soil microbes during sample storage.

Though the conditions created by sampling sediment may create a unique setting for these bacteria to proliferate, isotope effects associated with this enzymatic activity are inherent in the oxidation process and not unique to the storage situation. Given that *n*-alkane degrading bacteria have been identified in terrestrial, freshwater and ocean settings (Nie et al., 2014), this process may also affect the isotopic composition of *n*-alkanes before they are incorporated into the geological record. These changes in stable isotope values are potentially a complicating factor for interpretations of paleoenvironmental data from *n*-alkane δD and $\delta^{13}C$ data. However, this complication can be identified in samples with low odd-over-even predominance and minimized by freezing samples soon after collection.

Changes in the stable isotopic composition of *n*-alkanes prior to and during burial can fundamentally alter paleoclimatic inferences based on isotope results. Our data show that storage protocol can significantly impact measured *n*-alkane $\delta^{13}C$ and δD values. There are many different approaches to relate isotopic composition to paleoclimatic conditions which may be biased to a different extent based on the isotope-climate transfer function utilized. For example, a recent study (Magill et al., 2013a) utilized the $\delta^{13}C$ of ~2 million year old sedimentary *n*-alkanes to reconstruct the percentage of woody cover during a critical time period in human evolution. If we apply this same approach to $\delta^{13}C$ *n*-C₃₁ from Lusakert Cave to infer changes in ecosystem from these sample horizons we find that altered samples in Set B would overestimate tree cover by 20% compared to those in Set A (Fig 4a). The changes in δD values in *n*-C₃₁ would result in changes of reconstructed δD_{ppt} values (after Magill et al., 2013b) of up to 25‰ (Fig 4b). Such a

large difference would be misinterpreted as a major shift in precipitation source, temperature, amount of precipitation or elevation. For example, δD_{ppt} from the nearby Central Anatolian Plateau shows an adiabatic lapse rate for δD of 20‰ per 1000m (Schemmel et al., 2013). The offset between the δD values of sets A and B could be interpreted as transport path elevation differences of >1 km.

Conclusion

Storage of terrestrial sediments in ambient conditions for a period of three years resulted in the increase of short- and medium-chain length *n*-alkanes and enrichment of the hydrogen isotopic composition of long-chain length *n*-alkanes by up to 25‰. Carbon isotopes of long-chain length *n*-alkanes decrease by up to 4‰ during storage. DNA data show an increase in genera capable of breaking down *n*-alkanes, particularly *Aeromicrobium* and *Rhodococcus*. Both of these organisms have been identified to contain multiple coding regions for alkane monooxygenases *AlkB* and *CYP153*. The isotopic shift in hydrogen and carbon during storage is associated with a decrease in odd-over-even predominance and average chain length of the long chain *n*-alkanes. Our data show that post-depositional microbial processes have the potential to shift the stable isotopic composition of compounds thought to be generally resistant to isotopic alteration at temperatures below 150 °C. However, isotopic shifts are associated with a change in oxygen availability and moisture following sampling. These results highlight potential complications in utilizing sediments stored in non-sterile and ambient conditions, particularly sediment cores. In the absence of proper sediment sample storage protocols, resulting isotopic data from *n*-alkanes (δD and $\delta^{13}C$) cannot be used as reliable proxies for past environmental conditions.

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Figures

Fig 1: Relative abundances of *n*-alkanes from *n*-C₁₈ to *n*-C₃₃ in four paired sedimentary samples collected and frozen immediately (Set A) and four samples from identical locations stored for three years (Set B) from Lusakert Cave, Armenia.

Fig 2: Comparison of changes in odd-over-even predominance (Δ OEP) of samples during sample storage to changes in δ D values of *n*-C₂₉ (A) and *n*-C₃₁ (B) as well as changes in $\delta^{13}\text{C}$ values in *n*-C₂₉ (C) and *n*-C₃₁ (D)

Fig 3: Changes in microbial communities of sediments during sample storage. Total percent of bacterial genera present in samples with: coding regions for both *AlkB* and *CYP153* (A), multiple coding regions for *AlkB* (B), and multiple coding regions for *CYP153* (C)

Fig 4: Reconstruction of percent woody cover (A) (after Magill et al 2013a) and $\delta\text{D}_{\text{ppt}}$ (B) (after Magill et al 2013b) from *n*-C₃₁ in samples from Set A and Set B at Lusakert Cave

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Table 1. Concentration (µg/g) of *n*-alkanes in extracted sediments

Sample	A				B			
	1	2	3	4	1	2	3	4
<i>n</i> -C ₁₈	2	4	7	0	76	22	8	4
<i>n</i> -C ₁₉	3	5	8	0	48	19	12	17
<i>n</i> -C ₂₀	13	12	10	1	72	56	40	17
<i>n</i> -C ₂₁	16	15	10	3	156	169	115	56
<i>n</i> -C ₂₂	14	17	10	20	342	363	208	94
<i>n</i> -C ₂₃	30	17	24	20	420	213	87	189
<i>n</i> -C ₂₄	25	15	22	7	288	125	52	111
<i>n</i> -C ₂₅	107	59	30	47	180	81	35	67
<i>n</i> -C ₂₆	32	20	22	16	66	31	17	28
<i>n</i> -C ₂₇	254	170	105	160	198	81	52	61
<i>n</i> -C ₂₈	36	36	30	31	36	19	17	28
<i>n</i> -C ₂₉	532	507	321	418	456	238	144	150
<i>n</i> -C ₃₀	30	36	36	25	30	19	17	22
<i>n</i> -C ₃₁	505	568	306	357	432	331	167	144
<i>n</i> -C ₃₂	13	11	10	7	48	38	17	11
<i>n</i> -C ₃₃	109	54	49	34	102	69	29	17
LC µg/g*	1401	1299	781	969	1188	719	392	372
Total µg/g	1722	1546	1000	1147	2950	1872	1018	1015

*Long-chain (LC) *n*-alkane concentration = *n*-C₂₅ + *n*-C₂₇ + *n*-C₂₉ + *n*-C₃₁ + *n*-C₃₃

503 **Table 2.** Alkane abundances, δD values ($n\text{-C}_{29}$ and $n\text{-C}_{31}$, ‰), and $\delta^{13}\text{C}$ values ($n\text{-C}_{27}$ – $n\text{-C}_{33}$, ‰) for samples in Sets A and B.

Set	Sample	Abundances		δD		$\delta^{13}\text{C}$			
		OEP	ACL	$n\text{-C}_{29}$	$n\text{-C}_{31}$	$n\text{-C}_{27}$	$n\text{-C}_{29}$	$n\text{-C}_{31}$	$n\text{-C}_{33}$
A	1	11.1	29.2	–185	–191	–34.8	–36.1	–36.1	–36.3
	2	11.6	29.5	–186	–197	–30.1	–31.2	–32.7	–32.8
	3	6.7	29.4	–176	–182	–28.7	–30.7	–32.8	–32.9
	4	11.7	29.2	–191	–200	–31.8	–33.0	–32.9	–33.2
B	1	2.9	27.7	–166	–166	–34.3	–35.9	–36.3	–36.2
	2	3.5	28.2	–172	–185	–33.6	–35.4	–35.6	–36.1
	3	3.5	28.4	–168	–182	–33.1	–31.9	–35.4	–34.8
	4	2.2	27.1	–170	–182	–31.7	–33.1	–35.1	–34.1
Change During Storage*		OEP	ACL	$n\text{-C}_{29}$	$n\text{-C}_{31}$	$n\text{-C}_{27}$	$n\text{-C}_{29}$	$n\text{-C}_{31}$	$n\text{-C}_{33}$
	1	–8.2	–1.5	19	25	0.6	0.2	–0.2	0.2
	2	–8.1	–1.3	14	12	–3.5	–4.2	–2.9	–3.3
	3	–3.2	–1.0	8	0	–4.4	–1.2	–2.6	–1.9
	4	–9.5	–2.1	21	18	0.1	–0.2	–2.2	–0.8

504 *Change during storage was calculated by subtracting samples in Set B from those in Set A.