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Stable carbon and hydrogen isotopic fractionation of individual *n*-alkanes accompanying biodegradation: evidence from a group of progressively biodegraded oils

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Abstract

Seven crude oils of known source and maturity, representing a natural sequence of increasing degree of biodegradation, were collected from reservoirs in the Liaohe Basin, NE China, in an effort to determine the magnitude and direction of isotopic shift of carbon and hydrogen in individual *n*-alkanes during microbial degradation. The results show that biodegradation has little effect on the carbon isotopic composition of the whole oil. However, a sequential loss of *n*-alkanes leads to ^{13}C depletion of the bulk residual saturate fraction. The stable carbon isotopic compositions of aromatics and macromolecular organic matter (resins and asphaltenes) follow a pattern, with an overall trend towards ^{13}C enrichment of 0.8–1.7‰ in the residues. The stable carbon and hydrogen isotope values of individual *n*-alkanes demonstrate that they follow different trends during biodegradation. No significant carbon isotopic fractionation occurs for *n*-alkanes during slight to moderate biodegradation. However, there is a general increase of up to 4‰ in the $\delta^{13}C$ values of low molecular weight *n*-alkanes (C_{15}–C_{18}) during heavy biodegradation. In contrast, no isotopic fractionation occurs for higher molecular weight *n*-alkanes ($\geqslant C_{19}$). The conservative character of ^{13}C concentration for high molecular weight *n*-alkanes during biodegradation indicates that these compounds are effective tracers for source identification of heavily biodegraded oils. The hydrogen isotope compositions of *n*-alkanes show a significant fractionation as moderate biodegradation proceeds, resulting in an enrichment in D of up to ~35‰ upon heavy biodegradation. The dynamic fractionation in the stable carbon and hydrogen isotopes of *n*-alkanes accompanying biodegradation opens the possibility of quantitative estimation of the extent of biodegradation in subsurface petroleum reservoirs by introducing an isotopic kinetic modelling approach.

1. Introduction

Biodegradation of crude oil in the reservoir is an important process of secondary alteration with major economic consequences. Although the actual processes involved (e.g., site and rate of degradation, nutrient supply and nature of by-products) during in-reservoir biodegradation of crude oil remain obscure, their effects on the composition and physical properties are now well known (e.g., Connan, 1984; Peters and Moldowan, 1993; Larter et al., 2003; Head et al., 2003). In most cases, the components of crude oil are

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degraded sequentially in the order of *n*-alkanes > monocyclic alkanes > alkyl benzenes > isoprenoid alkanes > alkyl naphthalenes > bicyclic alkanes > steranes > hopanes (Peters and Moldowan, 1993 and references therein). Based on this sequence, many compound-related indicators have been proposed for the characterization of the level of biodegradation of crude oil. The terms "slight", "moderate", "heavy" and "severe" were defined and are usually used in the literature (Volkman et al., 1983; Peters and Moldowan, 1993; Wenger et al., 2001). However, it is not simple to estimate the fraction of oil lost quantitatively through degradation. Using data on bulk oil composition, variation in the concentration of degradation-resistant compounds and oil-charging biodegradation models, Larter et al. (2003) recently suggested that oils exhibiting heavy levels of biodegradation have typically lost up to 50% of their mass of C_{6+} components. The strong modification of the molecular fingerprints and parameters in severely degraded samples makes the source identification of crude oils using molecular profiles inconclusive or ambiguous (Peters and Moldowan, 1993).

Determination of the stable carbon and hydrogen isotopic compositions of oil at the molecular level is rapidly becoming a powerful tool for the accurate definition of petroleum systems through better constrained oil–oil and oil–source correlation (e.g., Schoell and Hayes, 1994; Li et al., 2001). However, to be considered as a reliable tool for correlation, the molecular isotopic composition of hydrocarbons must remain traceable through the various processes involved during hydrocarbon generation, migration, accumulation and in-reservoir alteration. The effects of biodegradation on the isotopic composition of individual compounds have been investigated recently for potential applications in petroleum and environmental science (Boreham et al., 1995; Mansuy et al., 1997; Huang et al., 1997; Wilkes et al., 2000; Hunkeler et al., 2001; Masterson et al., 2001; Mazeas et al., 2002; Pond et al., 2002; George et al., 2002; Mancini et al., 2003). It has long been recognized that the stable carbon isotopic compositions of natural gas components (C_2–C_5) is highly fractionated during biodegradation (James and Burns, 1984; Pallasser, 2000). Recent studies by Masterson et al. (2001) and George et al. (2002) showed that the effects of slight and moderate biodegradation on light hydrocarbons (C_5–C_9) also consistently lead to enrichment in ^{13}C for each remaining compound. However, Boreham et al. (1995) observed that slight and moderate biodegradation caused insignificant enrichment in residual C_{10}–C_{14} *n*-alkanes, with a maximum $\delta^{13}C$ increase of 0.5‰ (i.e., within analytical uncertainty). On the other hand, laboratory investigations using bacterial incubation also demonstrated that the stable carbon isotopic compositions of *n*-alkanes (>C_{12}) do not seem to be modified by biodegradation (Huang et al., 1997; Mansuy et al., 1997; Mazeas et al., 2002), thereby supporting field observations.

It is well known that *n*-alkanes are almost completely degraded, and thus undetectable in gas chromatograms after heavy biodegradation. Due to difficulties in effectively extracting *n*-alkanes from crude oil that has experienced heavy biodegradation, all the work reported to date is limited to investigation of the effects of slight and moderate biodegradation on the isotopic composition of individual *n*-alkanes. It has not been well established whether the isotopic compositions of *n*-alkanes would be affected by heavy biodegradation. Rogers and Savard (1999) predicted that the effects of severe biodegradation should result in less negative carbon isotopic values for C_{13}–C_{27} *n*-alkanes than in the case of no or slight to moderate biodegradation. Regardless of the concentration changes, if the isotopic composition of *n*-alkanes was not significantly affected, source apportionment could still be achieved by compound specific isotope analysis. This would be useful in practice for source identification of severely biodegraded oils, which is still difficult to determine unambiguously using traditional methods.

It is also of interest to investigate the effect of biodegradation on the stable hydrogen isotopic compositions of individual compounds. Compound-specific hydrogen isotope measurement only has recently become available (Burgoyne and Hayes, 1998; Hilkert et al., 1999). Because of the larger relative mass difference between deuterium and hydrogen than for ^{13}C and ^{12}C, hydrogen isotopes become fractionated significantly more during natural processes (Faure, 1986). Previous aerobic laboratory experiments on oils with up to moderate biodegradation showed that the C_{15}–C_{18} *n*-alkanes that were degraded at the fastest rates had the largest overall isotopic fractionation (~12–25‰ deuterium enrichment). On the other hand, the hydrogen isotopic compositions of the longer chain *n*-alkanes (C_{19}–C_{27}) were relatively stable during slight to moderate biodegradation (<5‰ overall deuterium enrichment), indicating that these compounds might be effective tracers for oil–source correlation (Pond et al., 2002). This is an important result with respect to laboratory investigations, but it is more important for petroleum geochemists to know if any fractionation takes place during natural biodegradation in subsurface petroleum reservoirs.

This paper aims to determine the magnitude and direction of carbon and hydrogen isotopic changes in individual *n*-alkanes during microbial degradation using a natural progressive biodegradation sequence of oils with identical source and maturity. Specifically, our work focusses on whether heavy biodegradation causes

significant carbon and hydrogen isotopic fractionation for C_{14}–C_{29} *n*-alkanes.

2. Samples and experimental conditions

2.1. Sample background

The sample suite comprises seven progressively biodegraded crude oils from the Ciyutou oilfield in the north Eastern Depression of Liaohe Basin, NE China (Table 1 and Fig. 1). The Liaohe basin is a Mesozoic–Cenozoic sedimentary basin and its petroleum geological setting has been well summarized elsewhere (Ge and Chen, 1993). Briefly, the Eastern Depression is a NE-trending trough situated between the central uplift and eastern uplift of the basin [Fig. 1(a)]. The Tertiary sequence in the depression includes the Paleogene Fangshenpao Formation, the Shahejie and Dongying Formation and the Neogene Guantao Formation. The Shahejie Formation in the basin can be subdivided into four lacustrine members (Es_4–Es_1, from bottom to top), with the Es_4 member being absent from the Eastern Depression. The Es_1 and Es_3 members are considered to be the main petroleum source units in the depression. Because the Es_1 member in the north Eastern Depression is presently immature to marginally mature (less than 0.5% *Ro*; Wu, 1993), the Es_3 member is likely to be the only effective source rock there (Sun, 1999). Petroleum migration and accumulation in this area are typically controlled by multiple-stage fault movements, which resulted in the formation of a number of small faulted block reservoirs at different depths [Fig. 1(b)].

2.2. Sample preparation

Asphaltenes were removed by precipitation with *n*-hexane followed by filtration. The de-asphaltened oils were then separated into saturate, aromatic and polar (NSO) fractions using column chromatography with a mixture of pre-activated silica gel and alumina (9:1, v/v) as stationary phase, with *n*-hexane, benzene, and ethanol as eluents, respectively. The saturate fraction was further separated into straight chain and branched/cyclic fractions using urea adduction. Each sample was added to a urea-saturated methanol solution and allowed to stand for 3 h in a freezer for complete adduction. The urea crystals were rinsed several times with cold *n*-pentane and dried under a nitrogen stream. The normal alkanes were recovered by dissolving the crystals in distilled water and extracting with *n*-pentane. For heavily biodegraded oils, 150–200 mg of saturate fraction was used. Due to the evaporative loss of light hydrocarbons from crude oils (C_6–C_{13}) during sample collection, storage and experimental, the following analyses only focus on the middle to high molecular weight range (C_{13}–C_{35}).

Table 1
Bulk characteristics and molecular parameters of oil samples[a]

Sample	Depth (m)	API°	Reservoir temperature (°C)	Gross composition (%)				Pr/Ph	Pr/*n*-C_{17}	P1	P2	P3	P4	Biodegradation level
				Saturate	Aromatic	Resins	Asphaltene							
IS006	1693–1734	12	45.6	44.6	27.2	14.2	9.3	n.a.	n.a.	0.11	0.56	0.47	0.88	Heavy
IS011	1776–1832	n.a.	n.a.	44.7	26.5	16.5	7.4	n.a.	n.a.	0.11	0.57	0.48	0.97	
IS009	1879–1902	14	60.0	50.9	22.3	16.0	8.8	2.1	4.4	0.10	0.59	0.45	0.94	Moderate
IS004	1939–1962	19	n.a.	47.8	21.9	18.5	6.7	2.0	2.9	0.11	0.57	0.43	0.97	
IS007	2139–2172	n.a.	60.3	54.8	16.2	13.7	2.5	2.5	3.0	0.09	0.59	0.38	1.06	Slight
IS002	2348–2367	38	66.8	77.2	8.1	8.5	4.8	3.6	0.6	0.12	0.58	0.39	1.03	None
IS005	2376–2423	38	65.2	68.3	10.7	8.7	4.2	3.3	0.7	0.10	0.57	0.40	1.04	

[a] P1 = 18α(H)-trisnorneohopane/17α(H)-trisnorhopane (T_s/T_m); P2 = C_{31} homohopane 22S/(22S + 22R); P3 = 20S/(20S + 20R) C_{29} 14α(H), 17α(H) steranes; P4 = C_{27} regular steranes/C_{29} regular steranes; n.a.: not available. Oil biodegradation level was determined based from scale of Wenger et al. (2001).

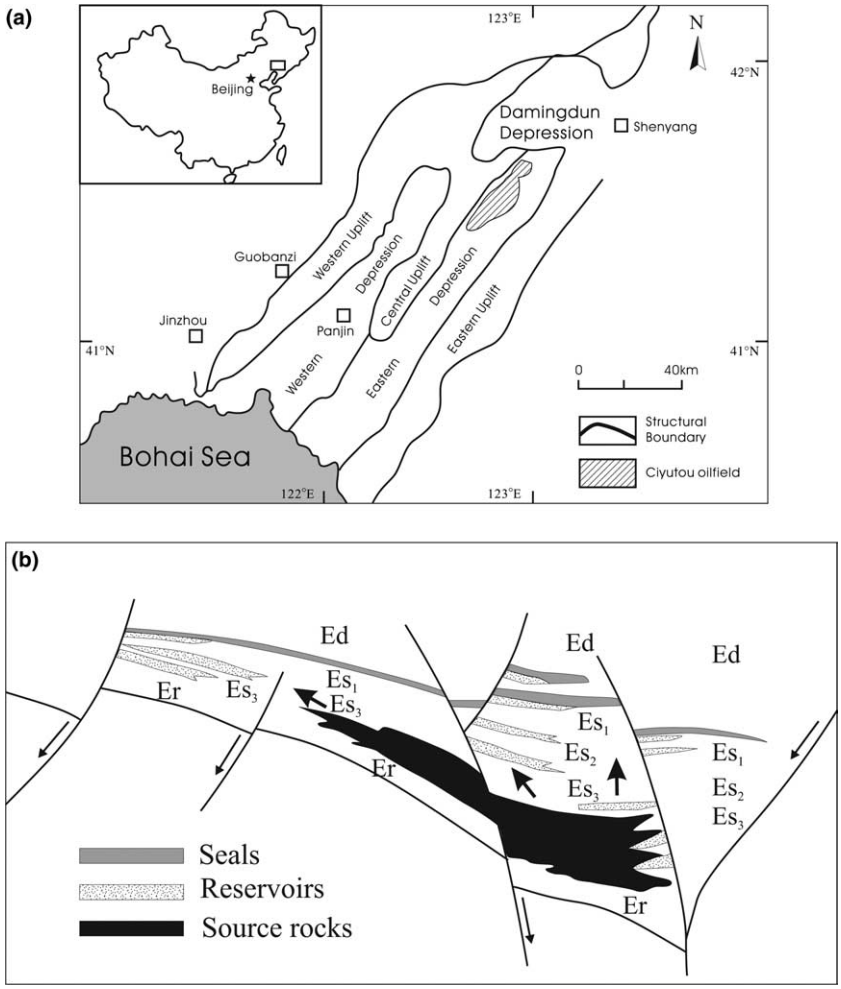

Fig. 1. Sketch maps showing the location of study area (a) and the fault system and source–reservoir–seal relationship (b).

2.3. Gas chromatography (GC)

Saturate fractions were analyzed using a Hewlett–Packard 6890 gas chromatograph equipped with a 30 m × 0.32 mm i.d. fused silica capillary column coated with a 0.25 μm film of HP-5. The temperature started at 60 °C, was held isothermally for 2 min and was then increased to 290 °C at 3 °C/min, followed by a 30 min hold at 290 °C. The carrier gas was nitrogen at a flow rate of 1.0 ml/min.

2.4. Gas chromatography–mass spectrometry (GC-MS)

Saturate fractions were analyzed using a Micromass Platform II spectrometer coupled to a Hewlett–Packard 6890 gas chromatograph. Chromatographic separation was achieved using a 30 m × 0.32 mm i.d. fused silica capillary column coated with a 0.25 μm film of DB-5MS (Chrompack). The oven temperature started at 65 °C (5 min) and was increased to 290 °C at 3 °C/min, followed by a 15 min hold. Helium was used as carrier gas at a flow rate of 1.0 ml/min. The transfer line temperature was 250 °C and the ion source temperature was 200 °C. The ion source was operated in the electron ionization (EI) mode at 70 eV. Full scanning was used to identify the biomarker compounds and calculate molecular parameters. The scanning range was from *m*/*z* 50–580.

2.5. Compound-specific isotope analysis

Stable carbon isotope analysis of *n*-alkanes and isoprenoids was performed using a VG Isochrom II system interfaced to a Hewlett–Packard 5890 gas chromatograph. The GC was fitted with a fused silica column (OV1, 50 m × 0.32 mm i.d.) leading directly into the

combustion interface. The initial temperature of the GC oven was 70 °C, at which it was held isothermally for 5 min and then programmed to 290 °C at 3 °C/min, followed by a 30 min hold. Helium (12 psi) was used as carrier gas. The injection of samples was conducted in the split mode at a ratio of 35:1. For calibration, CO_2 reference gas, calibrated against charcoal black (a national calibrated standard, with a value of −22.43‰ based on the PDB standard) was automatically introduced into the spectrometer in a series of pulses before and after the array of peaks of interest. The isotope values were calibrated against the reference gas and are reported in the usual δ notation relative to PDB.

Hydrogen isotopic analysis of individual *n*-alkanes was performed using a GC/TC/IRMS system comprising an HP6890 GC connected to a Finnigan MAT Delta^{plus}-XL mass spectrometer via a GC-C III interface. The GC was coupled with a capillary column (HP-5MS, 30 m × 0.32 mm × 0.22 μm) leading directly into a thermal conversion (reducing glassy carbon) interface. The oven temperature programme was from 50 to 90 °C at 15 °C/min (held 3 min) and 90 to 300 °C at 5 °C/min, followed by a 15 min hold. Helium was used as carrier gas at a flow rate of 1.5 ml/min (constant flow mode). The injection of samples was conducted via a cold on-column injection system. Individual *n*-alkanes separated by GC were instantaneously converted to H_2 at 1440 °C in a pyrolysis reactor (Burgoyne and Hayes, 1998; Hilkert et al., 1999). The H_3 factor was determined daily using standard hydrogen gas introduced through a GC-C III interface. Isodat software was used to correct for the H_3 factor. The mass spectrometer was tuned to ensure an H_3 factor of ~8–10; the daily variability was <0.3. Series of hydrogen reference gas pulses before and after the array of peaks of interest were injected via the GC-C III interface into the MS for the computation of δD values of sample compounds.

2.6. Reproducibility and accuracy of isotope data

The accuracy and the reproducibility of GC/IRMS data are mainly affected by chromatographic resolution, the presence of unresolved complex mixtures (UCMs) and background induced by column bleeding. The *n*-alkanes were purified using urea adduction and a low bleed capillary column was used. The accuracy of the data was routinely monitored using a set of standards of known isotopic composition before and after sample analysis. Briefly, the stable carbon isotope data were evaluated by analyzing a mixture of *n*- and isoprenoid alkanes with known $\delta^{13}C$ values from Geolab Norway. The stable hydrogen isotope data were tested using a mixture of *n*-alkanes with known δD values acquired from Indiana University, USA (C_{16}–C_{30}). Normally, one injection of standards was performed for every five sample injections. Each sample was analyzed at least two times, and the standard deviation (1σ) of the replicates was calculated for each *n*-alkane to estimate reproducibility. For carbon isotope data (Table 2), samples, analyzed in duplicate, had an average standard deviation of ~0.1–0.3‰; hydrogen isotope data analyzed in triplicate usually had a standard deviation within 3‰ and a maximum up to 4.5‰. For heavily biodegraded oils, compound-specific δD values were available only for a few *n*-alkanes (Table 3).

3. Results and discussion

3.1. Bulk oil characteristics

As shown in Table 1, crude oils collected from the Ciyutou oilfield have a wide range of API gravity, (12–38°).The C_{15+} gross chemical composition (% saturates, aromatic hydrocarbons, resins and asphaltenes; Table 1) show a progressive loss of saturated hydrocarbons from 77% to 45% among samples with decreasing API gravity, possibly indicating a progressive biodegradation sequence. In fact, the reservoir temperature of all the oils ranged from 46 to 67 °C, the best temperature range for bacterial activity in subsurface petroleum reservoirs. Previous oil–oil and oil–source correlations have revealed that hydrocarbon generation and migration in the Ciyutou oilfield occurred substantially near the end of Dongying deposition, over a relatively narrow time range (Chen and Li, 1998). It has also been suggested that heavy oils in the area were formed by biodegradation of normal gravity oils generated by mature Es_3 lacustrine source rocks due to the multiple stage fault movements during hydrocarbon generation, migration and accumulation (Chen and Li, 1998; Sun, 1999). As discussed below in Section 3.2, hopanes appear unaffected during biodegradation of the sample set. Similar T_s/T_m ratios and C_{31} homohopane 22S/(22S + 22R) ratios for the samples further support the idea that this sample group is derived from a single source kitchen, with expulsion over a narrow maturity range (Table 1).

3.2. Determination of level of biodegradation

Based on the alteration of biomarker compounds, several schemes have been proposed to rank the level of biodegradation of crude oil and these have been applied successfully in petroleum exploration (Volkman et al., 1983; Peters and Moldowan, 1993; Wenger et al., 2001). The scale of Wenger et al. (2001) was used as a framework in this study.

Because *n*-alkanes are usually the first compound class to be consumed by bacteria, biodegraded oils are easily recognized via GC from the absence or presence

Table 2
Stable carbon isotopic composition of oils and C_{15+} fractions (‰)

Sample	$\delta^{13}C_{whole\ oil}$	$\delta^{13}C_{saturate}$	$\delta^{13}C_{aromatic}$	$\delta^{13}C_{resins}$	$\delta^{13}C_{asphaltene}$
IS006	−25.8	−27.5	−24.5	−23.8	−24.8
IS011	−25.8	−27.7	−24.6	−23.8	−24.6
IS009	−26.0	−27.5	−24.9	−24.2	−24.9
IS004	−26.0	−27.3	−24.9	−24.2	−25.1
IS007	−25.8	−26.5	−25.1	−24.8	−25.3
IS002	−25.9	−26.2	−25.3	−25.5	−25.9
IS005	−25.9	−26.3	−25.2	−25.2	−25.7

Table 3
Stable carbon and hydrogen isotope composition of *n*-alkanes from oils (‰)

ID	IS006		IS011		IS009		IS004		IS007		IS002		IS005	
	$\delta^{13}C$	δD	$\delta^{13}C$	δD	$\delta^{13}C$	δD	$\delta^{13}C$	δD	$\delta^{13}C$	δD	$\delta^{13}C$	δD	$\delta^{13}C$	δD
n-C_{14}											−25.1		−24.5	
n-C_{15}	−22.9								−25.4		−25.0		−24.3	
n-C_{16}	−23.3				−24.0			−170.9	−25.1	−180.3	−25.0	−178.9	−24.6	−183.6
n-C_{17}	−24.2	−153.6	−21.5		−24.4	−173.8	−24.3	−176.4	−24.7	−178.9	−25.3	−179.1	−24.8	−181.2
n-C_{18}	−24.5	−147.9	−23.4		−25.1	−173.5	−25.5	−178.3	−25.6	−177.5	−25.8	−177.4	−25.4	−178.7
n-C_{19}	−25.3	−143.6	−25.2	−152.6	−24.9	−171.6	−25.7	−177.0	−25.6	−176.1	−25.8	−175.6	−25.4	−176.8
n-C_{20}	−25.5	−142.3	−25.1	−152.7	−25.1	−166.9	−25.6	−172.9	−25.9	−171.0	−25.9	−176.2	−25.4	−173.5
n-C_{21}	−25.6	−141.4	−25.5	−150.5	−25.7	−166.9	−25.8	−172.7	−26.0	−171.1	−26.1	−176.3	−25.9	−173.3
n-C_{22}	−25.8	−140.2	−26.2	−150.0	−26.1	−166.2	−25.8	−170.5	−25.2	−172.1	−26.1	−177.1	−25.9	−174.2
n-C_{23}	−26.0	−139.2	−26.2	−150.9	−26.2	−163.2	−26.1	−169.7	−25.7	−170.3	−26.2	−170.8	−25.9	−172.3
n-C_{24}	−26.0		−26.4	−152.7	−26.3	−161.9	−26.0	−164.6	−25.4	−162.1	−26.2	−170.8	−25.5	−166.7
n-C_{25}	−25.6		−26.4	−150.4	−26.5	−164.2	−26.1	−167.6	−25.5	−163.4	−26.3	−173.7	−25.7	−166.0
n-C_{26}	−26.3		−26.7	−149.1	−26.8		−26.5	−161.4	−25.9	−160.3	−26.4	−164.2	−25.9	−164.7
n-C_{27}							−26.6	−164.9	−25.8		−26.5	−166.8	−26.2	−163.3
n-C_{28}							−26.4		−26.3		−26.4		−26.2	
n-C_{29}							−26.1		−26.4		−26.8		−26.7	
Pristane					−26.8		−25.5		−25.9		−25.1		−24.9	
Phytane					−27.2		−26.9		−26.4		−26.3		−26.1	

in very low concentration of *n*-alkanes and the presence of an UCM (Connan, 1984). As shown in Fig. 2, *n*-alkanes are progressively depleted in crude oils with increasing extent of biodegradation. In the shallowest and most degraded oils that have the lowest API gravity, both *n*-alkanes and isoprenoid alkanes have been completely removed as shown from the GC chromatograms, resulting in saturate fractions comprised largely of branched and cyclic alkanes.

Several molecular parameters were calculated to investigate the effects of biodegradation on the various compound classes and further help to define the level of biodegradation. Samples IS002 and IS005 are considered to be non-biodegraded and are characterized by a full range of *n*-alkanes (Fig. 2) and high concentrations of saturate fractions (77.2% and 68.3%, respectively, Table 1). No significant change in hopanoid-related parameters and the partial depletion of steranes (Table 1, Fig. 3) indicate that the level of biodegradation in the remaining sample set ranges from slight to heavy on the scale of Wenger et al. (2001).

As shown in Table 1 and Fig. 3, selective biodegradation of the steranes has occurred. Preferential removal of the C_{27} homologue and ααα 20R isomer results in a decrease in the $\sum C_{27}$ steranes/$\sum C_{29}$ steranes ratio and an increase in the 20S/(20S + 20R) ratio. This confirms previous observations of the removal in the order $C_{27} > C_{28} > C_{29}$ steranes and selective removal of the 5α 20R C_{29}-sterane compared to the 20S epimer (Rullkötter and Wendisch, 1982; McKirdy et al., 1983). Samples IS006 and IS011 show almost complete loss of *n*-alkanes and acyclic isoprenoids and significant biodegradation of steranes, indicating these two oils have reached the "heavy biodegraded" level on the scale of Wenger et al. (2001). All steranes and hopanes appear intact and low molecular weight *n*-alkanes ($<C_{17}$) are still seen clearly within sample IS007 (Table 1, Fig. 2), indicating that this sample has only reached the "slight"

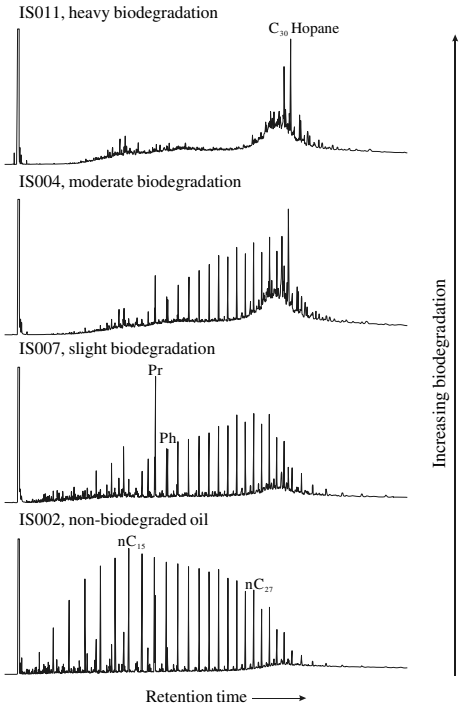

Fig. 2. Representative gas chromatograms of saturated hydrocarbons as a function of extent of biodegradation.

level of biodegradation. Almost complete loss of low molecular weight *n*-alkanes ($<C_{17}$) and slight degradation of steranes suggest samples IS004 and IS009 are moderately degraded.

Normally, the *n*-alkanes would be degraded prior to significant alteration of other compound classes, resulting in higher pristane/*n*-C_{17} ratios for slightly to moderately biodegraded oils than their non-biodegraded counterparts (Winters and Williams, 1969). Although the pristane/*n*-C_{17} ratio increases with biodegradation (Table 1), pristane and phytane appear to be partially degraded in this sample set. A decrease in pristane/phytane ratio with increasing biodegradation suggests that pristane is possibly degraded faster than phytane. This suggestion is further supported by the stable carbon isotopic fractionations of these two compounds, as shown below. Some loss of isoprenoids prior to complete removal of the *n*-alkanes was expected, but is rarely reported (Peters and Moldowan, 1993). The present data suggest that caution should be taken when the pristane/phytane ratio is used to correlate slightly to moderately biodegraded oils.

3.3. Bulk isotope dynamics accompanying biodegradation

Stable carbon isotopic compositions of compound-grouped fractions can be altered by biodegradation

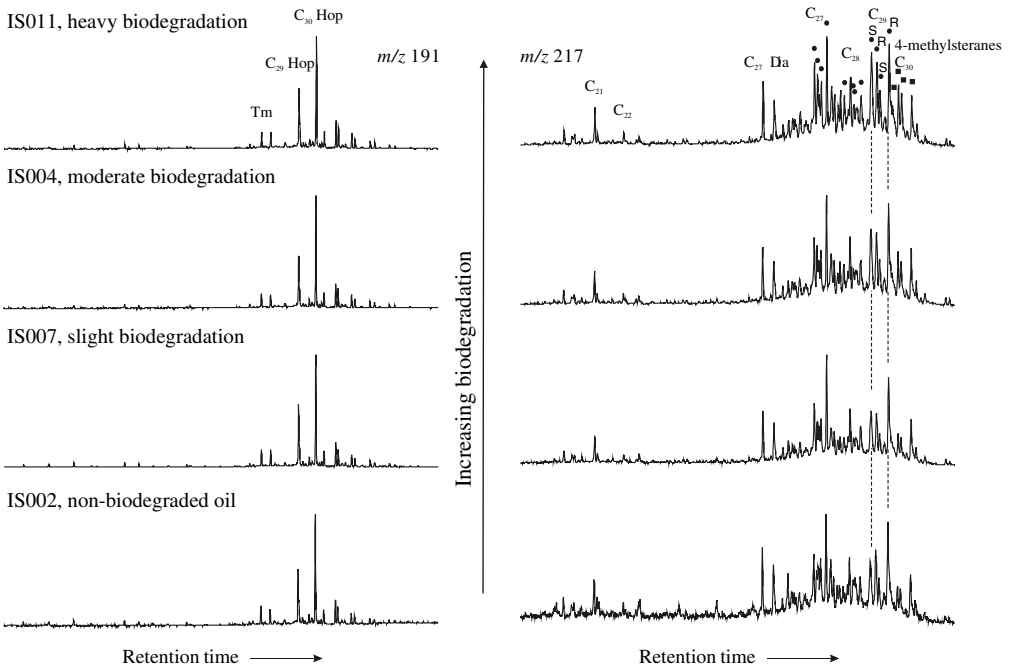

Fig. 3. Hopane and sterane distributions corresponding to extent of biodegradation as revealed by *m/z* 191 (left) and 217 (right) mass chromatograms. No significant change is apparent in hopanes, but selective removal of the 20R 5α-C_{29} sterane relative to its 20S epimer during the course of the biodegradation is clearly visible. ●, steranes, ■, 4-methylsteranes, S, 20S 5α-C_{29} sterane, R, 20R 5α-C_{29} sterane.

(Stahl, 1980; Momper and Williams, 1984; Sofer, 1984; Connan, 1984). As shown in Table 2 and Fig. 4, the stable carbon isotopic compositions of the whole oil samples remain unchanged during biodegradation. This appears to be a useful tool for screening heavily biodegraded oils, as the analysis is rapid and does not require any sample preparation. Similar observations were also reported in the case of the Exxon Valdez oil spill even for residues sampled 5 years after the spill (Kvenvolden et al., 1995).

The stable carbon isotopic composition of the oil fractions does not follow a consistent trend (Fig. 4). The $\delta^{13}C$ values of the aromatics, resins and asphaltenes show a trend towards ^{13}C enrichment during biodegradation, in agreement with most previous observations (Connan, 1984). However, the $\delta^{13}C$ values of the saturate fractions demonstrate a trend to more negative values as biodegradation proceeds, due to variation in source input. Hence, multiple source contributions to individual *n*-alkanes are evident in their $\delta^{13}C$ values and the shape or slope of these values as a function of carbon number. For example, oils derived from carbonate source rocks with marine organic matter typically show a ^{13}C enrichment with increasing molecular weight so that loss of light hydrocarbons would lead to an increase in the bulk $\delta^{13}C$ value of the residual saturate fraction (Chung et al., 1994). The maximum isotopic shift in the bulk saturate fraction of a marine oil with the complete loss of *n*-alkanes is about 2‰ (Palmer, 1984; Sofer, 1984). In contrast, terrestrially-derived oils usually have a steeply dipping $\delta^{13}C$ profile toward more negative values for high molecular weight *n*-alkanes (Murray et al., 1994). Therefore, the loss of low molecular weight hydrocarbons would lead to a decrease in the bulk $\delta^{13}C$ value of the residual saturate fraction. As shown in Fig. 4, the slight biodegradation with partial depletion in low molecular weight *n*-alkanes in sample IS007 results in little change in the bulk $\delta^{13}C$ value of the residual saturate fraction, but significant change in carbon isotopic composition occurs in moderately to heavily biodegraded oils (Fig. 2). This trend implies that compounds remaining in the saturate fractions are more depleted in ^{13}C than the *n*-alkanes. GC and GC/MS analyses revealed that hopanes are the main components in the saturate fractions of heavily biodegraded oils, which elute over an enhanced UCM background (Fig. 2). The $\delta^{13}C$ values of hopanes in these oils range from −40‰ to −60‰ (Xiong, 1997), suggesting that the relative enrichment in hopanes is one of the primary causes for the isotopic shift in the saturate fractions.

3.4. Isotope fractionation in individual n-alkanes

The $\delta^{13}C$ and δD values of individual *n*-alkanes from the progressive biodegradation sequence are summarized in Table 3 and Figs. 5 and 6, respectively.

As shown in Fig. 5, the $\delta^{13}C$ values of the high molecular weight *n*-alkanes ($\geqslant C_{19}$) remain constant, even for the heavily biodegraded samples. However, there is a systematic increase in the $\delta^{13}C$ values of the lower molecular weight *n*-alkanes. For slight biodegradation with partial depletion of low molecular weight *n*-alkanes in sample IS007, these alkanes still show almost identical $\delta^{13}C$ values to those of the unaltered samples (i.e., IS002 and IS005). With a large depletion in low molecular weight *n*-alkanes corresponding to moderate biodegradation (samples IS004 and IS009), there is a small carbon isotopic fractionation (~0.5‰ toward less negative $\delta^{13}C$ values). As the standard deviation for our isotope analysis is generally within ±0.3‰, such a difference should be a real

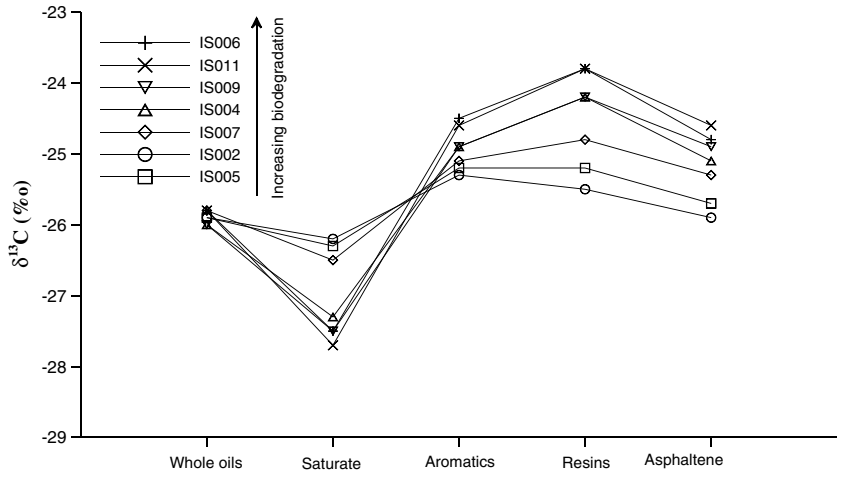

Fig. 4. Stable carbon isotopic compositions of whole oils and C_{15+} fractions from a natural progressive biodegradation sequence.

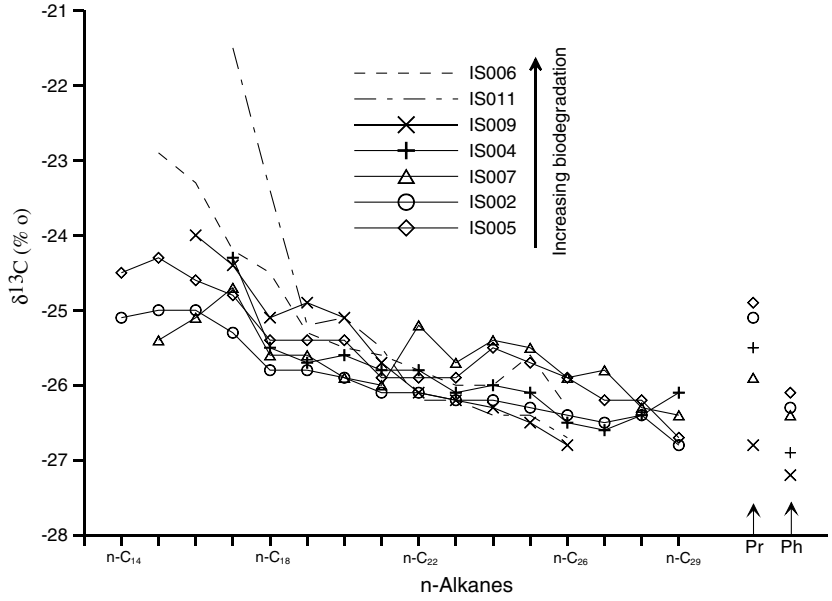

Fig. 5. Stable carbon isotopic profiles of *n*-alkanes from a natural progressive biodegradation sequence.

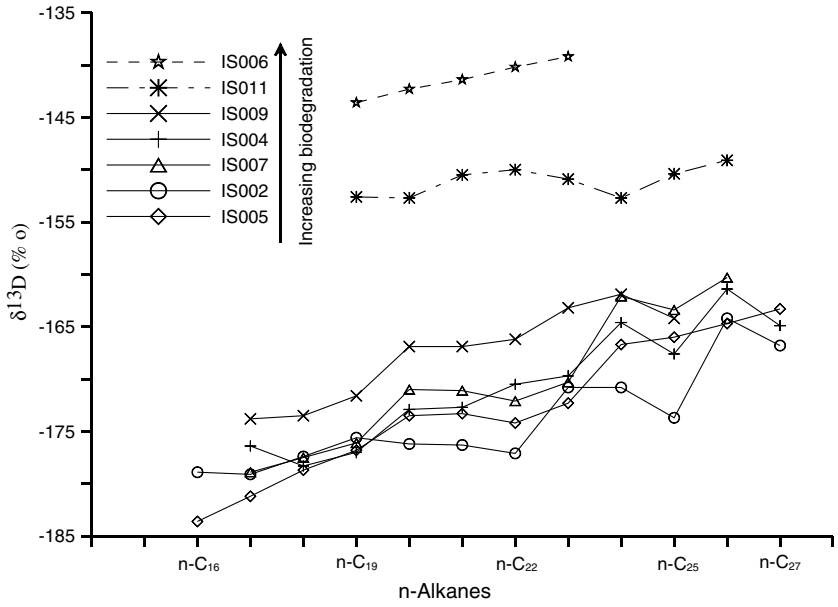

Fig. 6. Stable hydrogen isotopic profiles of *n*-alkanes from a natural progressive biodegradation sequence.

reflection of the biodegradation effect. This result is supported by previous field observations that show up to a 0.5‰ increase in the $\delta^{13}C$ values for the residual C_{10}–C_{14} *n*-alkanes after slight to moderate biodegradation(Boreham et al., 1995). For the heavily biodegraded samples (IS006 and IS011), the almost complete depletion in low molecular *n*-alkanes is accompanied by a significant carbon isotopic fractionation (up to 4‰) toward less negative $\delta^{13}C$ values than those of the non-biodegraded oils (Table 3; Fig. 5).

Degradation of pristane (Pr) and phytane (Ph) is generally considered to be negligible when *n*-alkanes are still available as a substrate (Seifert and Moldowan, 1979). However, it is obvious that pristane and phytane are degraded before *n*-alkanes are completely eliminated.

Table 1 shows a decrease in Pr/Ph ratio with increasing biodegradation, suggesting a higher rate for pristane degradation than phytane, as it is impossible for phytane to be produced during biodegradation. Theoretically, if pristane and phytane are affected during the early stage of oil degradation, the effect should be reflected in their carbon isotopic compositions. Thus, the $\delta^{13}C$ value shifts for pristane and phytane during biodegradation could potentially provide another line of evidence for this conclusion. Five samples representing non- to moderately biodegraded oils were analyzed to investigate the carbon isotopic fractionation of pristane and phytane. The measurements show a systematic shift in the isotopic composition as biodegradation proceeds, ranging from −25.0‰ to −26.8‰ and −26.2‰ to −27.2‰ for pristane and phytane, respectively (Table 3 and Fig. 5). Interestingly, degradation of pristane is accompanied by a much larger carbon isotopic fractionation than that of phytane. Because the fractionation associated with biodegradation is a function of the extent of biodegradation (as discussed later), the difference in carbon isotopic fractionation between pristane and phytane further supports the idea that pristane is degraded faster than phytane.

Figure 6 displays the fractionation in hydrogen isotope composition of *n*-alkanes in the biodegradation sequence. As the hydrogen isotopic composition of low molecular weight *n*-alkanes ($<C_{16}$) may be affected by evaporation (Wang and Huang, 2003), the following discussion is limited to the C_{16} to C_{27} *n*-alkanes. Indeed, a large variation was observed in the δD of individual *n*-alkanes during biodegradation (−184‰ to −139‰). The δD values of individual *n*-alkanes remain unchanged during slight biodegradation, but begin to show effect as moderate biodegradation occurs. Although the average isotopic shift is only about 5‰ during moderate biodegradation (within the analytical uncertainty), the overall trend is clear, and could reflect the true biodegradation effect. Interestingly, there is no hydrogen isotopic fractionation in *n*-alkanes observed in sample IS004 that is moderately biodegraded. As this sample is less degraded than sample IS009 based on molecular parameters in Table 1, this result possibly indicates a moderate level of biodegradation as the starting point for hydrogen isotopic fractionation of *n*-alkanes, and heavy biodegradation would lead to greater hydrogen isotope fractionation (up to 35‰). These results are in contrast with those obtained previously from laboratory experiments where no clear hydrogen isotope fractionation was noted for long chain *n*-alkanes (C_{19}–C_{27}) during biodegradation (<5‰ in overall deuterium enrichment; Pond et al., 2002).

Theoretically, stable carbon and hydrogen isotope fractionations of organic compounds accompanying biodegradation are strictly controlled by kinetic isotope effects during initial transformation. Usually, the reaction rate is slightly faster for molecules with light isotopes (^1H, ^{12}C) as compared to molecules with heavy isotopes (^2H, ^{13}C) and, as a result, the residual compound becomes increasingly enriched in the heavy isotopes as the biodegradation proceeds, similar to Rayleigh distillation. The extent of isotope fractionation depends on the biodegradation processes and elements involved (carbon or hydrogen), and is often proportional to the relative mass difference between the two isotopes of a given element. This explains the significant carbon and hydrogen isotopic fractionation of individual *n*-alkanes, but not the carbon isotope dynamics of isoprenoid alkanes that show increasingly negative $\delta^{13}C$ values with increasing biodegradation.

Anaerobic biodegradation accounts for hydrocarbon degradation in most petroleum reservoirs and has only recently been widely recognized as an important mechanism (Rueter et al., 1994; Zengler et al., 1999; Widdel and Rabus, 2001; Larter et al., 2003). Methane carbon isotopic data were obtained on gas samples associated with both non-biodegraded and biodegraded oils in the study area (Table 4). The results show isotopically lighter methane in biodegraded reservoirs, indicating that methanogenesis, an indicator of anoxic conditions,

Table 4
Stable carbon isotopic composition of methane collected from associated reservoirs

Sample	Stratum	Depth (m)	δ^{13} C (‰)	Reservoir
99LH38-86	Es_3	2247–2288	−41.8	Non-biodegraded
99LH40-88	Es_1	2225–2253	−41.1	
99LH23-32	Ed	1935–2076	−40.7	
99LH23-19	Es_1	2120–2125	−40.3	
99LH52	Es_1	2654–2680	−40.7	
99LH8	Es_1	?–1552	−55.4	Biodegraded
99LH38-198	Es_3	1983–2017	−54.3	
99LH32-98	Es_1	2009–2022	−47.1	
99LH13	Ed	1793–1827	−48.5	

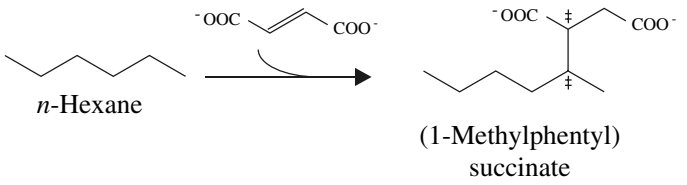

Fig. 7. Pathway showing initial reaction during anaerobic degradation of hexane. Double daggers mark chiral carbon atoms. This pathway is a radical reaction of with fumarate, yielding substituted succinates, and represents the most common activation mechanism detected in several physiological types of anaerobes (after Widdel and Rabus, 2001).

was involved in biodegradation. It is known that anaerobic *n*-alkane activation occurs via the addition of a carbon chain compound, and that the site in the *n*-alkane chain for such addition is changeable. Although different sites of initial attack on *n*-alkanes are possible, the *n*-alkanes are mainly activated at C-2 in connection with addition of fumarate, yielding succinate (Fig. 7; Aeckersberg et al., 1998; So and Young, 1999). Therefore, the kinetic isotope effect associated with biodegradation of *n*-alkanes mainly represents a site-specific isotope fractionation effect associated with C-2. As compound-specific isotopic analysis averages the values for all the carbon atoms in an *n*-alkane, any isotopic shift that results from the C-2 site-specific isotope fractionation upon biodegradation chain shortening will be isotopically buffered when a longer carbon chain is present. This hypothesis is corroborated by the variation in the $\delta^{13}C$ values of *n*-alkanes with increasing molecular weight and chain length (Fig. 5).

If the biodegradation process indeed follows the Rayleigh model, an exponential isotopic enrichment or depletion would be expected in the residual reactant reservoir as the product is removed, which can be expressed as

$$\delta = (\delta_i + 1000)F^{(\alpha-1)} - 1000,$$

where F is the fraction of the *n*-alkane remaining in the sample, δ is the isotopic value of individual *n*-alkane at a particular F, δ_i is the initial value of individual *n*-alkane and α is the fractionation factor. Rearrangement of the above equation gives

$$\ln[(\delta + 1000)/(\delta_i + 1000)] = (\alpha - 1)\ln F.$$

Plotting ln $[(\delta + 1000)/(\delta_i + 1000)]$ vs. ln F should result in a straight line with a slope of $(\alpha - 1)$.

Because of the relatively small carbon isotopic fractionation for the higher molecular-weight *n*-alkanes (Fig. 5), we only calculated the carbon isotopic fractionation for C_{17} *n*-alkane. The result shows that the carbon isotopic fractionation factor (α) for C_{17} *n*-alkane is 0.98, indicating a single biodegradation step is responsible for this fractionation. In the same way, we made calculations for hydrogen isotopic fractionation on the C_{17} and C_{22} *n*-alkanes. This showed that the observed hydrogen isotopic fractionation does not fit with the theoretical model. There is poor correlation between ln $[(\delta + 1000)/(\delta_i + 1000)]$ and ln F during the course of the biodegradation for nC_{17} ($R^2 = 0.70$) and nC_{22} ($R^2 = 0.36$). One possibility is that changes in δD values are correlated with biodegradation, but are not only caused by biodegradation. For example, it is possible for isotopic exchange to occur at different reservoir temperatures over a long geological history. On the other hand, little is known about organic chemical mechanisms of hydrogen abstraction/oxidation for *n*-alkanes during biodegradation. Hydrogen isotopic exchange of one or more organic hydrogen atoms during chemical transformation is another possibility. The larger hydrogen isotope fractionation of both low and high molecular-weight *n*-alkanes observed in this study possibly indicates that the involvement of hydrogen atoms is more complicated than for carbon during microbial degradation.

3.5. Geochemical implications

Because biodegradation can alter commonly used maturity and correlation parameters, it is necessary to develop geochemical parameters which are independent of biodegradation (Peters and Moldowan, 1993). A few studies have shown that stable carbon isotopic compositions of *n*-alkanes are not affected by biodegradation and thus can be used to correlate biodegraded oils with their source (e.g., Boreham et al., 1995; Mansuy et al., 1997; Huang et al., 1997; Mazeas et al., 2002). However, most published work limited investigation to a moderate level of biodegradation due to the difficulty of *n*-alkane recovery from heavily biodegraded oils. It has not been well established whether the isotopic compositions of compounds of interest (e.g., *n*-alkanes) would be affected by more severe biodegradation. The present study provides a robust base for compound specific analysis as a tool for correlating a heavily biodegraded oil with its source by using the conservative characteristic of $\delta^{13}C$ values of high molecular weight *n*-alkanes ($\geqslant C_{19}$). The hydrogen isotopic composition of *n*-alkanes is more sensitive to biodegradation than carbon isotope composition because of the larger

relative mass difference between deuterium and hydrogen compared to ^{13}C and ^{12}C and more complicated processes are possibly also involved during microbial degradation. Therefore, the use of *n*-alkane δD values as an oil/oil and oil/source correlation tool is currently limited to petroleum reservoirs with non-biodegraded to slightly biodegraded oils.

On the other hand, measurement of the stable carbon and hydrogen isotopic fractionations of *n*-alkanes accompanying biodegradation also opens up the possibility of quantitative assessment on the extent of biodegradation using a Rayleigh model if the petroleum reservoir in question is a relatively closed system.

4. Conclusions

1. For terrestrially sourced oils, biodegradation has little effect on the carbon isotopic composition of the whole oil. While progressive biodegradation leads to a sequential loss of *n*-alkanes, resulting in a bulk ^{13}C depletion for the residual saturate fraction, the carbon isotopic compositions of the residual aromatic hydrocarbons, resins and asphaltene fractions are relatively enriched in ^{13}C by 0.8–1.7‰.
2. No significant isotopic fractionation occurs for carbon isotopes of *n*-alkanes during slight to moderate biodegradation. Likewise, the carbon isotopic composition of higher molecular weight *n*-alkanes ($\geqslant C_{19}$) remains constant during severe biodegradation. In contrast, there is a dramatic change in the $\delta^{13}C$ values of low molecular weight *n*-alkanes (C_{15}–C_{18}) during heavy biodegradation, which is expressed as an increase in $\delta^{13}C$ value by up to 4‰ relative to unaltered oils.
3. Degradation of pristane and phytane occurs as *n*-alkanes are rapidly biodegraded. Decreasing Pr/Ph ratios and the difference in carbon isotopic shifts experienced by the remaining pristane and phytane suggest that pristane is degraded faster than phytane.
4. During moderate to severe biodegradation, residual *n*-alkanes show enrichment in deuterium by up to ~35‰.
5. The conservative character of $\delta^{13}C$ of high molecular weight *n*-alkanes indicates that these compounds are effective tracers for source identification in heavily biodegraded reservoirs. The larger relative mass difference between deuterium and hydrogen compared to ^{13}C and ^{12}C indicates that *D*/*H* ratios of *n*-alkanes are more sensitive to biodegradation than $^{13}C/^{12}C$ ratios. Thus, the use of δD values of *n*-alkanes for oil/oil and oil/source correlation in biodegraded oils has more limited diagnostic potential than the use of $\delta^{13}C$ values.
6. Stable carbon and hydrogen isotopic fractionations of *n*-alkanes during biodegradation provide the possibility of quantitatively estimating the extent of biodegradation using isotopic kinetic modelling.

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