Paper I

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Acidic compounds in biodegraded petroleum

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Abstract

Twelve oil samples have been characterised by titration, FT-IR and chromatographic analysis to determine the differences between the organic acid composition of biodegraded and non-biodegraded oils. The biodegraded oils have higher total acid and total base contents, both by titration and extraction. The molecular weight ranges of the extracted acids are lowest in the biodegraded oils, and the equivalent weight calculations indicate a dominance of multi-functional molecules. Gel permeation chromatography gives a molecular weight range with most of the molecules between 300 and 500 g/mol. FT-IR shows that the extracted acids from biodegraded oils are more carboxylic and aliphatic while the non-degraded oils are more phenolic. Molecular analysis of the derivatised extracts give UCM envelopes for biodegraded oils, and no molecular identification. The results indicate that the acidic constituents in biodegraded oils are a product of the biodegradation, as the composition is very different from the non-biodegraded oils.

1. Introduction

Microbial degradation of crude oil in the reservoir has for a long time been associated with increased acidity of the remaining oil phase (Behar and Albrecht, 1984; Wenger et al., 2002; Meredith et al., 2000). This has most often been investigated from an oil quality perspective, since acid oils are a considerable problem in terms of corrosion (e.g., Slavcheva et al., 1999). The standard industrial measure is the total acid number, TAN, determined by non-aqueous titration (ASTM664-89) which gives the acidity as mg base needed to neutralise the acids in 1 g oil. This procedure does not give information on the molecular composition of the acids, though it is possible to estimate strong acid concentrations relative to weaker acids. Recently, the usefulness of TAN measurements for estimating corrosion risks has been challenged, and the search for better acid characterisation method has been intensified in the downstream context (Slavcheva et al., 1999; Tomczyk et al., 2001).

Organic geochemistry has a long tradition of molecular analysis of carboxylic acids and phenols in crude oils, to a large degree focussed on two subjects: carboxylic acids as hypothetical intermediates in oil generation processes from lipids or other precursor materials (e.g., Cooper, 1962; Shimoyama and Johns, 1971), and the oil–water interactions of short-chain fatty acids and phenols (e.g., Barth and Riis, 1992; Taylor et al., 1997). The aim has been to describe the molecular composition and geochemical significance of specific types of

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acidic compounds. The relationship between the molecular composition and the total oil acidity has mostly not been considered, with the exception of Meredith et al. (2000), who used GC analysis of methyl esters of extracted acids to estimate the contribution from the carboxylic acids to the total acid number.

In petroleum chemistry, a wider range of acidic compounds has been investigated, especially with the use of mass spectrometric techniques (e.g., Seifert and Teeter, 1970; Tomczyk et al., 2001; Quian et al., 2001). In some cases, the analyses were performed on distillation fractions, including heavy residues, so the oil has been heated to a considerable temperature and reactions may have occurred. The results may therefore not be representative of crude oils. The risk of oxidation due to contact with air during production and refining is also considerable. However, even with these uncertainties, the range of compounds observed in the acid fractions is wide, and includes acidic nitrogen compounds like indoles, carbazoles and amide derivatives, sulphur compounds, and polyfunctional compounds. The early work of McKay et al. (1975), concludes that the acid compound types in Wilmington crude oil is 28% carboxylic acids, 28% phenols, 28% pyrroles (indoles and carbazols) and 16% amides.

Recent developments of mass spectrometry has given new information on the range of acidic structures found in petroleum. Tomczyk et al. (2001) reported on the acid type distribution in an oil from the San Joaquin valley. The oil is reported as aerobically biodegraded (TAN 5.19 mg KOH/g oil). The sample is the methylated acids recovered after liquid/liquid base extraction. The paper reports that 40% of the acid types are not carboxylic acids, and that only 10% of the acidic compounds contain two oxygens (as found in one carboxylic acid group), while approximately 50% of the acids contain nitrogen and 25% contain sulphur. Acidic sulphur compounds like thiols are easily lost because they oxidise in air. It is suggested that amino acids from the biomass of the micro-organisms could be the source of acidic components in the oil. Quian et al. (2001) have also reported results from high resolution mass spectrometry showing an acid distribution from C_{15} to C_{55}, with 1–6 naphthenic rings and 1–3 aromatic rings, and functional groups comprising O_2, O_3, O_4, O_2S, O_3S and O_4S. The acids were taken from a South America heavy crude oil by solid-phase extraction. These results show the complexity of the acidic fraction of crude oils, and suggest that the geochemical view of phenols and carboxylic acids comprising the major portion of the acidic species may be oversimplified.

Crude oils are also known to contain basic compounds, with basic nitrogen compounds comprising the most well known group. The total base content can be determined as the total base number, TBN, by a potentiometric titration procedure (ASTM2896-88). It is not usual to discuss the acids and bases in the crude oils together. However, acids and bases are connected through the acid–base equilibrium, conventionally written as (1).

$$\underset{\text{acid}}{HA} + : \underset{\text{base}}{B} \leftrightarrow \underset{\text{corresponding base}}{A^-} + \underset{\text{corresponding acid}}{H : B^+} \quad (1)$$

Depending on the balance between acids and bases in the oil, this equilibrium can be displaced to the right with excess acid or to the left with excess base, and the same molecular species can belong to the acidic fraction in one case and the basic fraction in the other case. The acids and bases may also exist as acid-base pairs which neutralise each other, or even as amphotheric molecules with both acidic and basic functionalities in the same structure, e.g., amino acids. It is therefore reasonable to consider acids and bases together when the crude oil acid-base properties are under investigation. The combined presence of acids and bases in the crude oil also explains why some oils may act as a buffer and neutralise both strongly acidic and strongly basic aqueous solutions.

Biodegraded oils are ''heavy'', i.e. with a generally higher density and viscosity than non-biodegraded oils. This may be caused by the relative increase in heavy components as the lighter hydrocarbons are removed by the microbial activity, or it may be due to production of heavy components, including acids, as products of the microbial processes or by degradation of dead biomass (Behar and Albrecht, 1984; Tomczyk et al., 2001; Watson et al., 2002). In the first case, the acid composition should not be significantly different in biodegraded and non-biodegraded oils. If, however, the ''new'' acids are products of metabolic processes, a difference between the geochemically produced acids and the microbially produced acids is to be expected.

In this work, we have determined the TAN and TBN of 12 carefully preserved oil samples, and correlated these properties with bulk oil properties with emphasis on the differences in acid composition between biodegraded and non-biodegraded oils. The samples come from four biodegraded reservoirs (seven samples) and five reservoirs where no degradation is observed. Acidic compounds have been extracted with aqueous/ethanolic sodium hydroxide solution, and the recovered acids characterised by FT-IR and gel permeation chromatography for determining the molecular weight range of the extract. Gas-chromatographic analysis combined with mass spectrometry (GC-MS) has been used in an attempt at molecular identification, but with limited success in identification of individual components in the biodegraded crudes. The combined data are used to establish a picture of the molecular types that may be part of the acidic fraction of the oils. The source of the acids can then better be evaluated, and the properties of acidic biodegraded oils can be better understood,

both as a geochemically important process and in terms of predicting corrosion risks.

2. Experimental

2.1. Samples

The oil samples were supplied by Norsk Hydro. 0.5–1 L samples that had been stored in closed, refrigerated sample containers were transferred to aluminium flasks (to store the samples in darkness), and the TAN determined immediately. Aliquots of the oil were removed from this stock bottle after heating to 60 °C and homogenisation to ensure a representative composition of each sample.

2.2. TAN and TBN titration

A Metrohm autotitrator (model 798 MPT Titrino) connected to a Metrohm Solvotrode combined LL pH glass electrode (model 6.0229.100) was used for the determination of acid and base numbers.

The acid numbers, TAN, defined as the amount (mg) of potassium hydroxide (KOH) necessary to titrate 1 g sample to a well-defined inflection point, were determined according to ASTM664-89. This is a standardised potentiometric titration with KOH in 2-propanol.

The base numbers, TBN, also defined as the amount (mg) KOH necessary to titrate 1 g sample to a well-defined inflection point, were determined by the standard procedure ASTM2896-88 with modifications according to Dubey and Doe (1993). This is a potentiometric titration procedure using perchloric acid dissolved in acetic acid as titrant and methylene isobutylketone as solvent for the crude oil samples.

Both procedures involve daily standardisation of unstable KOH solutions and determination of blank values. The determination of the blank value was simplified through the use of standard curve intercept values. The volumes of KOH solution necessary to titrate a given amount KHFT (potassium hydrogen phthalate) to a well-defined inflection point are measured as a function of amount of KHFT. The plot of the volume KOH used as a function of mass KHFT should comprise a straight-lined plot, from which the KOH concentration and blank value can be determined from the regression line. The blank value from this procedure has a higher accuracy than in the original ASTM procedure, and by simply checking the validity of the standard-curve with one or two titrations each day, the blank value is determined.

For both TAN and TBN determination, at least three parallel titrations of the crude oil samples were made, using 5–20 g oil in each titration. The results are generally associated with an error of less than 4% for values of TAN exceeding 1 mg KOH/g oil, but errors as large as 20% could be observed for very low TANs. The TBN values are generally associated with less uncertainty than the TAN values. For all values the TBNs were determined within an error of approximately 5%.

Selected samples were also re-titrated after acidification in the following way: after the usual TAN titration was complete, an excess of a strong acid, trichloroacetic acid, (concentration ca. 0.1 M) was added to protonate all acid anions (corresponding bases in Eq. (1)), and the titration was repeated. This procedure will register all compounds with acidic properties, independently of their initial state as protonated acids or neutralised anions.

2.3. Asphaltene content and density determination

The asphaltenes were precipitated by refluxing a portion of the oil with a 40 times excess of hexane for 6 hours. The precipitated asphaltenes were removed by filtration through a Whatman GF/C glass fibre filter, and dissolved in dichloromethane (DCM). The concentration of the asphaltenes in the DCM solution was determined gravimetrically, by deposition of 10 μl solution on the weighing pan of a Cahn electrobalance (range 0.0001–2 mg), letting the solvent evaporate, and noting the weight of the non-volatile residue after 20 min.

Some of the asphaltene and maltene fractions were titrated to determine the distribution of the titrable acids after the fractionation.

The density of the crude oil was determined at 20 °C using an Anton Paar DMA60 densitometer connected to an Anton Paar DMA602HT measuring cell.

2.4. Extraction of the acid fraction

A 100 g portion of the crude oil was diluted with 100 ml hexane and extracted with 1 M sodium hydroxide (NaOH) in 50% ethanol, 50% water in a procedure previously described (Costantinides and Arich (1967) and Hoeiland et al. (2001)). The extraction was repeated three times. Even with careful shaking, many of the oils formed stable emulsions that had to be left to separate for up to three days. The extracts were pooled, the pH adjusted to 2 with concentrated hydrochloric acid, and the acids back-extracted with three portions of dichloromethane and dried with sodium sulphate. The acid extract was concentrated by evaporation with N_2(g) and the yield determined gravimetrically as described for the asphaltenes.

Some of the acid fractions and the remaining acid stripped oils were titrated to determine the efficiency of the extraction.

2.5. FT-IR of the acid fractions

A drop of a sample solution was deposited on potassium bromide powder, and dried to remove the solvent. The FT-IR spectra were collected by the diffuse reflectance technique using a Nicolet 800 FT-IR instrument.

2.6. Gel permeation chromatography of the acid fraction

Gel permeation chromatography was performed on a PL-gel 3 μl MIXED-E column using tetrahydrofuran as the mobile phase at a rate of 0.5 ml/min. The detector used was a Sedex 55 light scattering detector (S.E.D.E.R.E, France), which is linearly sensitive to all compounds with a boiling point above 100 °C. The molecular weight calibration was based on representative standards covering a range of molecular weights from 122 g/mol (benzoic acid) to 599 g/mol (vanadyl-octaethyl porphyrin), with the addition of polystyrene standards at 30,000 and 70,000 g/mol for the higher molecular weights. A linear relationship between the logarithm of the molecular weight of seven standard compounds and the retention time (RT) was observed with a correlation coefficient of 0.98.

2.7. GC-MS of hydrocarbons and derivatised acids

Before the GC-MS analysis of the hydrocarbon fraction for evaluation of biodegradation, polar components in the oil were removed by eluting the crude oils through Supelclean™ Envi™ Chrom P SPE Tubes from SUPELCO using a solute containing 25% DCM and 75% hexane.

GC-MS analysis of the acid extracts was done after methylation with diazomethane in ether solution to methylate carboxylic acids and phenols. The acid fractions were derivatised with diazomethane by the following procedure. All solvents were removed from an aliquot of the sample in a stream of nitrogen, and the sample was re-dissolved in a minimum amount of methanol. A solution of diazomethane in diethyl ether (0.05–0.1 M) was added until no more nitrogen was generated, the sample glass was tightly corked and left for 30 min. The sample volume was then reduced to 1 ml, and an internal standard (*n*-hexadecane) was added before GC-MS analysis.

The samples were analysed on a GC-MSD (HP 5890-II with HP Auto 5890) and a 25 m WCOT fused silica column (CP-Sil_8_CB) equipped with both FID and HP5971 MSD. The run started at 50 °C with 3 °C/min to 70 °C and 6 °C/min up to 320 °C. The final temperature was held for 10 min. The injector temperature was 300 °C, the FID was at 350 °C and the MSD had a temperature of 280 °C.

The degree of biodegradation was estimated according to the Peters and Moldowan (1993) biodegradation scale using data from the hydrocarbon fraction chromatogram.

3. Results

3.1. Samples and degree of biodegradation

The samples comprise 12 crude oils, mostly from the Norwegian continental shelf, as listed in Table 1. The biodegraded oils are numbered as B1–B4. From two of the fields, there are two different samples with similar, but not identical properties, given as **a** and **b**. Some large volume (bulk) sample is also included, though the sample handling may not have been as careful as for the other samples. In total, seven different samples of biodegraded oils have been analysed. Five non-biodegraded oils have been analysed for comparison, given as S1–S5 in the Table 1. Parallel analysis has been performed for some properties, and are given in the tables as (1) and (2) after the sample names.

GC-MS analysis of the hydrocarbon fractions has been used to determine the degree of biodegradation on the Peters and Moldowan (1993) scale. The results are given in Table 1. Oils S1–S5 showed no indication of biodegradation. For the biodegraded oils, the levels range from 2 for sample B1 (some *n*-alkanes left, pristane/*n*-$C_{17} > 1$) to 8 for sample B3 (very low levels of n-alkanes and isoprenoids, steranes removed and hopanes partly removed). The sample B4b shows conflicting trends, with presence of isoprenoids and some n-alkanes indicating biodegradation level 2, while the steranes have been removed and the hopanes partly removed indicating biodegradation level 8. Our interpretation is that this oil sample contains a mixture of a strongly biodegraded oil with a light, non-biodegraded oil. It comes from a field where the degree of biodegradation is known to vary laterally.

3.2. TAN, TBN and extractable acids

Table 1 shows the total acid number (TAN) and total base number (TBN) of all the oil samples, given in milligrams potassium hydroxide per g oil (mg KOH/g oil) and for the acids also as the concentration of acidic functionalities in millimoles acids per gram oil. The table also includes the density and asphaltene content of the oils.

The acid content of the oils clearly divide them into two groups, the biodegraded oils (B1–B4) with TAN numbers in the range 1–3 mg KOH/g oil and the non-biodegraded oils (S1–S5) with TAN values below 0.2 mg KOH/g oil.

The total base number, TBN, shows a similar tendency, but the difference between the two groups is smaller; biodegraded oils are in the range 1.1–4 mg KOH/g

Table 1
Properties of the oils

	Biodeg. level	TAN (mg KOH/g)	TAN (meq./g)	Extr. acids (mg/g oil)	TBN (mg KOH/g)	Asphaltene (mg/g oil)	Density (g ml)
B1(1)	2	2.4	0.042	1.77	2.79	14.136	0.940
B1(2)	2			2.83			
B1(bulk)	2	2.22	0.0396	4.66	3.425	17.2	
B2a	6	3.2	0.0571	5.37	1.06	3.083	0.914
B2b	6	2.66	0.0474	7.09			0.931
B3(1)	8	2.11	0.0376	4.55	4.14	25.37	0.931
B3(2)	8	2.90	0.0517	9.49			
B4a(1)	8	1.10	0.0196	2.63	1.21	3.785	0.895
B4a(2)				3.74			0.895
B4b(1)	8/2	1.51	0.0269	4.34	1.29	1.94	0.900
B4b(2)	8/2	1.92	0.0342	5.15		1.94	
S1	0	0.13	0.0023	1.543	0.94	2.734	0.850
S2(1)	0	0.04	0.0007	1.44	0.35	0.422	0.845
S2(2)	0			2.14			
S3	0	0.03	0.0005	1.343	0.72	2.172	0.850
S4	0	0.026	0.0005	0.185	0.15	0.692	0.791
S5	0	0.12	0.0021	1.24	0.47	1.431	0.845

Samples marked (1) and (2) are parallel determinations. Samples marked a and b are different samples from the same field and well. Biodeg. level: biodegradation level estimated as described in Peters and Moldowan (1993). TAN: total acid number, given both as mg KOH used per gram oil, and as equivalent acidic functionalities. Extr. acids: yield of extract, given as mg material recovered per gram oil. TBN: total base number from titration with perchloric acid.

Table 2
Correlations between oil properties in Table 1

	TAN	Extract	TBN	Asphaltene	Density
TAN	1	0.797	0.750	0.599	0.886
Extract		1	0.646	0.381	0.675
TBN			1	0.967	0.788
Asphaltene				1	0.657
Density					1

and non-biodegraded oils 0.15–0.95 mg KOH/g. The relative amounts of acids and bases vary in all proportions even in this limited sample set. Two biodegraded oils have excess base (samples from B1 and B3), two have excess acid (B2a and B4b) and the remaining oils are close to "neutral". The non-biodegraded oils all have a considerable excess of base.

The amounts of extractable acids, given as mg/g oil, is systematically higher in the biodegraded oils, but the difference between biodegraded and non-biodegraded oils is not as large as for the TAN. Co-extraction of polar compounds that are not acidic will result in a higher value of extract than the real acid content. This may explain the reduced difference in extract yields compared to the TAN values of the oils, and also the very high equivalent weights calculated from the extract yield and titration data for the low TAN oils, since this is the ratio of extract yields by weight and the molar concentrations of the acidic components.

Looking at all the bulk oil properties, the TAN and TBN are correlated with each other to a considerable degree ($R^2 = 0.75$) and with the oil density, see Table 2. The TAN and amount of acids extracted show only a slightly higher degree of correlation ($R^2 = 0.797$). The only combination with a very low correlation is the amount of acid extract and the asphaltene content.

3.3. The distribution of acids in oil fractions

For the oils B1, B2a and B4, the TAN of the asphaltenes and maltenes were separately determined after deasphalting of the oil. In B1 and B2, the asphaltenes contained a very little of the total acidic functionality. In B4 about 1/3 of the titrable acidity resided in the asphaltene fraction, while 2/3 was recovered in the maltenes (Table 3). The high TAN of the asphaltenes in this oil indicates a high content of acid functional groups. A simple calculation of the equivalent weights per acid functionality in the asphaltenes gives a value of 56 g/mol, which corresponds to a simplified average elemental distribution of one oxygen per three carbons

Table 3
TAN in oil fractions

TAN values (mg KOH/g oil)				TAN values		
Whole oil	Asphaltenes	Maltenes	% recovery	Extract backt.	Stripped oil	% Recovery
1.50	0.57	1.14	114			
2.22	0.09	2.2	104	0.72	0.05	29.16
2.66				0.72	0.05	29.16
3.20	b.d.	3.2	100		0.14	
1.92				0.83	0.28	57.90

Extract backt.: Re-titration of the acid extract.
b.d.: below detection level.

(C_3H_4O), if no other heteroatoms are considered as acidic sites. However, the other oils do not show a similarly high acid content in the asphaltene fraction.

Comparing the TAN of the fractions with the whole oil, the sum of the fractions is slightly higher than the original value (114%). The deasphalting procedure can have led to an increase in the acidity, e.g., by oxidation on contact with air, but the change is small and the uncertainty especially in the TAN determination of the asphaltene fraction is larger than for the crude oils, since it is based on a small amount of material.

The efficiency of the acid extraction was checked by titration of the extracts and the acid stripped oil (Table 3). This showed a more complex situation; very little acidity remained in the acid stripped oils, but the recovery of acidity in the extract was much lower than the original content, with recoveries of 29–57% of the whole oil TAN. To achieve the highest recoveries, acidification and re-titration of the extracts was necessary. Direct titration gave much lower TAN values, probably due to neutralisation of acidic functionalities in the back-extraction step of the procedure.

3.4. Equivalent weights and molecular weights

The molecular weight distribution in the acid extracts was determined by gel permeation chromatography (GPC) on a styrene–divinylbenzene column with a useful range up to 30 000 g/mol. The retention of smaller molecules in the pores of the gel slows them down relative to the larger molecules. The molecular weight range is shown as the spread of the peak on the *X*-axis of the chromatogram. The retention is calibrated to standards of known molecular weight. The method cannot take into account the possible effects of molecular geometry, which can influence the apparent molecular weights if compact, cyclic molecules are retained more in the pore volumes than chains with the same molecular weight. The deviations between observed and real molecular weight can be aggravated since polystyrene standards are used for calibrating the high molecular weight range. The calibration will thus be most correct for linear mol-

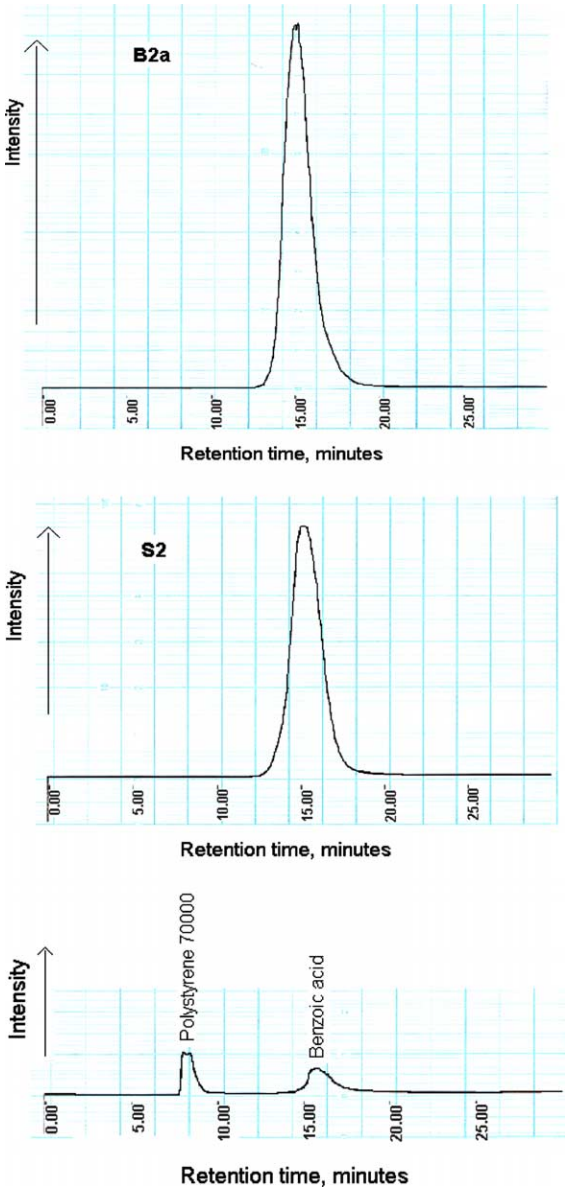

Fig. 1. GPC chromatograms.

ecules, while compact molecules may be more retarded and thus be assigned a lower molecular weight. Other potential problems include skewing of results towards the lower molecular weight range if the solvent strength of the mobile phase is not sufficient to completely suppress adsorption of sample components on the gels. The data should therefore be used with caution. However, the method gives information on the spread of molecular weights in the sample which is not available by average methods.

Examples of GPC traces are given in Fig. 1, which shows the acid extract from oils B2a and S2 together with a standard run. The standard shows how the heaviest compounds elute first, and the low molecular compounds at the end of the chromatogram. The peaks are narrow and more or less bell shaped, indicating the presence of a quite narrow range of molecular weights.

The results from the GPC chromatography are presented in Table 4, together with the average equivalent weights calculated by dividing the extract yields by the TAN values of the oils after conversion to a molar concentration scale. Two calculated values are given, the first assumes that all acidic compounds have been extracted, the second assumes a recovery of 33% where the reduction in acidity does not influence any of the other parameters (e.g., a simple neutralisation of the functional groups). The correct average equivalent weight should thus lie between these two values.

The GPC molecular distributions are given by the molecular weight of the start of the peak (GPC-max), the maximum peak intensity (GPC-med) and the end of the peak (GPC-min). The values are plotted relative to TAN in Fig. 2.

There is a clear reduction in the molecular weight range of the acids in the extract with increasing TAN,

Table 4
Equivalent weights and molecular weight ranges from GPC

	Extract (mg/g oil)	TAN (mg KOH/g)	Equiv. weight (g/mol)	Corr. equiv. wt. (g/mol)	GPC-min (g/mol)	GPC-med (g/mol)	GPC-max (g/mol)
B1(1)	1.773	2.41	41	124	70	370	1400
B2a	6.15	3.24	106	319	40	320	1130
B3	4.547	2.11	121	363	15	205	1800
B4a(1)	2.603	1.08	135	4.6	50	315	1400
B4a(2)	3.776	1.1	193	578	30	340	1350
B4a(3)	3.709	1.1	189	567	5	330	2100
B4b	4	1.51	149	446	15	160	1550
S1	1.543	0.13	666	1997	45	335	3850
S2(1)	1.44	0.044	1836	5507	20	320	2200
S3	1.343	0.03	2511	7533	60	345	1575
S4	0.185	0.026	399	1197	50	475	3450
S5	1.24	0.12	580	1739	60	325	1575

Equiv. weight: equivalent weight per acid functionality based on titration.
Corr. equiv. wt.: equivalent weight corrected for 1/3 recovery of acids.
GPC-min: lowest molecular weight observed in GPC trace.
GPC-med: molecular weight at peak maximum in GPC trace.
GPC-max: highest molecular weight observed in GPC trace.

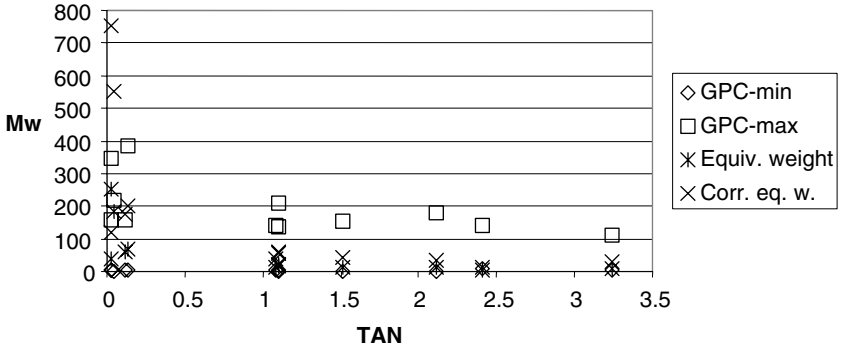

Fig. 2. Molecular weight and equivalent weight ranges relative to TAN.

especially noticeable for the maximum molecular weight by GPC. The biodegradation of the oils thus leads to a lower molecular weight distribution for the acids. The presence of very low equivalent weight values suggests that there is a considerable proportion of di- or poly-functional molecules. The data suggest that an average molecular weight of 300–500 g/mol is a reasonable assumption, but the GPC molecular weight range also includes compounds with molecular weights over 1000 g/mol.

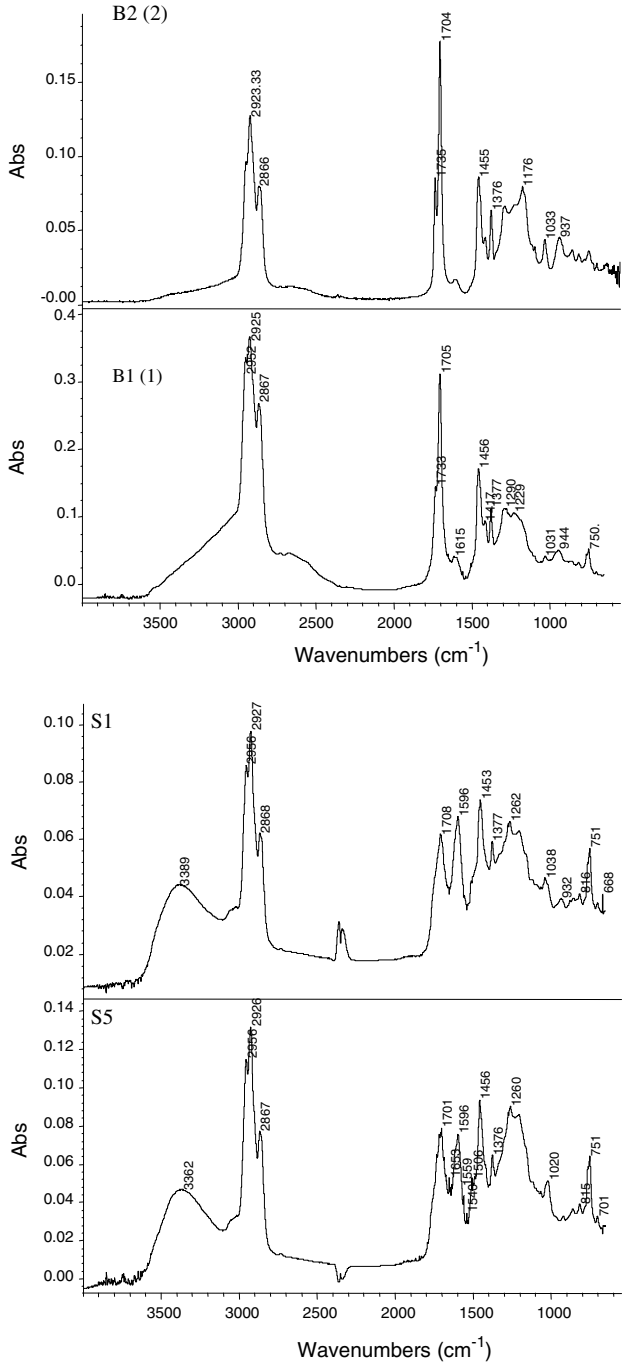

Fig. 3. FT-IR spectra of acid extracts of biodegraded and non-biodegraded oils.

Table 5
Peaks and intensities for FT-IR spectra of acid extracts

	·OH stretch	C–H Aro	CH-alkyl(1)	CH-alkyl(2)	CH-alkyl(3)	CH═O	C═O str. (1)	C═O str.(2)	C═C, –NH2	C–H bend	C–H bend	C–O and O–H		C–H bend
B1(1)	3500–3000	n.r.	2952	2926	2868	2668	1732 (sh)	1705	1608	1457	1377	1290	1229	750
	Slope		93.3%	100.0%	74.7%	12.4%	39.3%	87.1%	10.7%	46.1%	30.3%	30.9%	28.9%	12.9%
B1(2)[a]	3500–3000	n.r.	2951	2923	2866	2663	1731(sh)	1704	1613	1456	1377	1287	1230	750
	Slope		61.7%	81.3%	50.5%	9.3%	29.9%	100.0%	12.1%	48.6%	32.7%	35.5%	33.6%	16.8%
B2a(1)	3500–3000	n.r.	2952	2924	2868	2666	1735(sh)	1705	n.r.	1457	1377	1296	1229	743
	Slope		89.4%	100.0%	65.7%	8.3%	10.6%	89.4%		37.0%	25.0%	26.9%	22.2%	16.7%
B2a(2)[a]	3500–3000	n.r.	2950	2923	2866	n.r.	1734(sh)	1704	1609	1456	1376	1288	1177	751
	Slope		54.2%	71.8%	44.6%	0.0%	48.0%	100.0%	9.0%	48.0%	35.0%	37.3%	44.6%	20.9%
B3[a]	3500–3000	n.r.	2950	2923	2856	2676	1735(sh)	1705	1611	1456	1376	1286	1228	752
	Slope		53.1%	73.1%	43.8%	5.0%	28.1%	100.0%	7.5%	45.0%	29.4%	31.9%	30.0%	11.9%
B4b(1)	3412(W)	n.r.	2952	2926	2867	2730	1738	1716(sh)	1597	1456	1376	1265	1171	749
	11.5%		91.1%	100.0%	64.8%	3.8%	56.2%	41.4%	16.3%	46.2%	29.9%	36.1%	37.3%	15.7%
B4b(2)[a]	3333(W)	3021	2952	2924	2866	2654	n.p.	1705	1596	1456	1376	1290	1229	n.r.
	13.0%	19.8%	79.8%	100.0%	58.2%	8.9%		82.5%	16.4%	40.3%	23.9%	27.2%	25.4%	
S1	3390(W)	3052	2956	2927	2867	n.r.	1734(sh)	1700	1596	1457	1376	1275	1209	750
	49.4%	38.4%	95.0%	100.0%	67.6%		60.8%	77.4%	74.6%	74.6%	64.7%	73.7%	68.9%	65.2%
S2	3356(W)	3021	2965	2927	2868	n.r.	n.r.	1705	1596	1456	1377	1270	1204	750
	26.7%	14.3%	87.4%	100.0%	51.5%			25.4%	36.3%	58.0%	36.9%	48.5%	44.3%	59.3%
S3	3384(W)	3022	2955	2926	2867	n.r.	1736	1700	1596	1456	1378	1270	1202	751
	35.1%	24.7%	85.6%	100.0%	57.7%		25.8%	37.1%	52.6%	71.1%	14.6%	59.8%	56.7%	59.8%
S4	3431(W)	n.r.	2957	2926	2857	n.r.	n.r.	1705	n.r.	1457	1375	1275	1188	750
	23.4%		53.8%	64.4%	39.3%			48.4%		47.0%	41.0%	67.8%	53.3%	100.0%
S5	3385(W)	3027	2956	2926	2867	n.r.	1735(sh)	1701	1596	1456	1376	1275	1206	751
	35.4%	25.4%	87.7%	100.0%	58.5%		50.0%	74.6%	57.7%	71.5%	40.8%	69.2%	65.4%	49.2%

n.r.: not registered; n.p.: not present.

[a] Samples analysed later than the initial sample set. The relative intensities may not be the same as for the first sample set.

3.5. FT-IR analysis

Diffuse reflectance infrared spectroscopy of the extracted acids gives spectra that clearly separate the biodegraded and non-biodegraded oils. Fig. 3 shows examples of both oil types. The biodegraded oils typically have a slope with no clear maximum in the –OH, –NH– region of 3500–3000 cm^{-1}, which is typically seen for carboxylic acids There is a wide band around 2600 cm^{-1} that may also represent acids, or

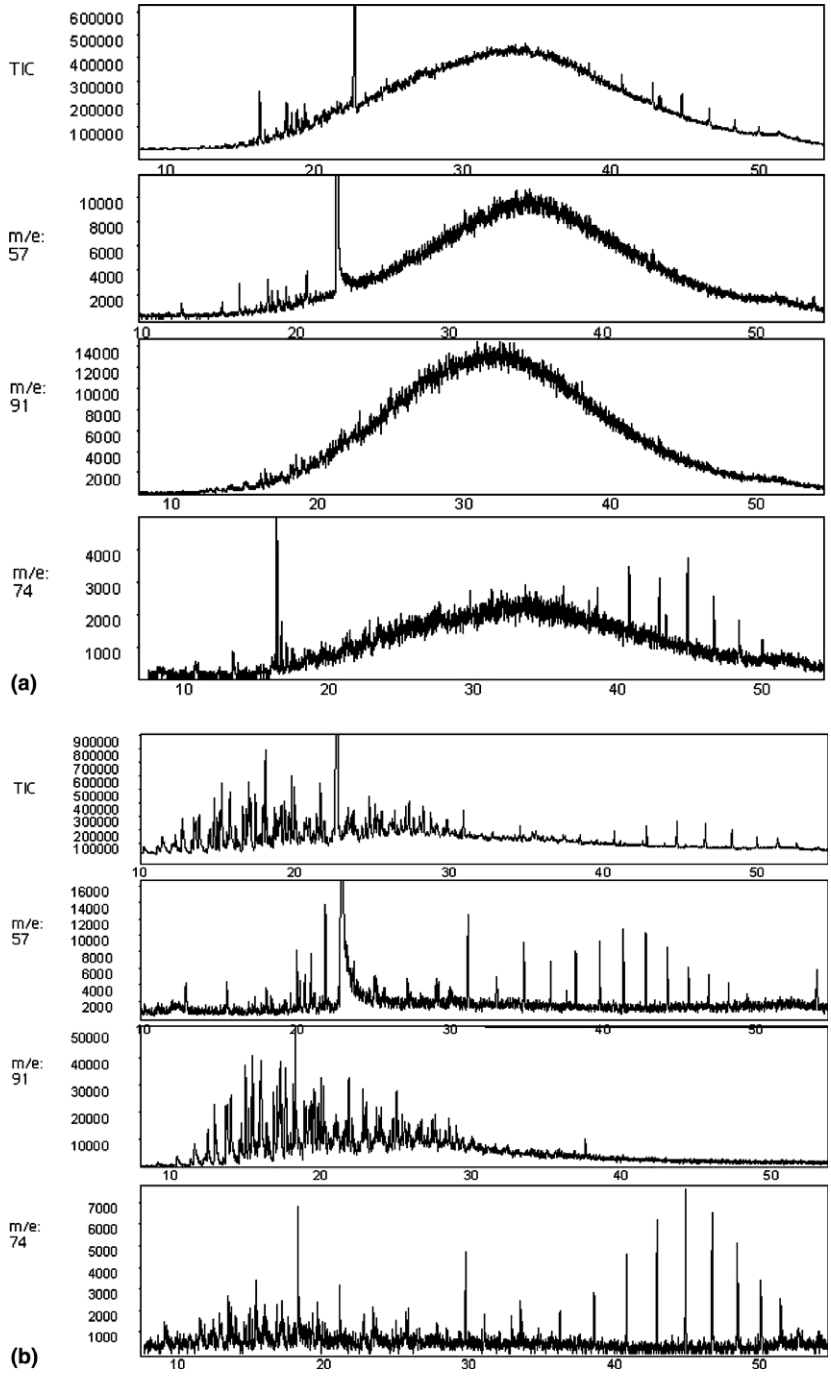

Fig. 4. GC-MS chromatograms. (a) Methylated acid extract from oil B2a. (b) Methylated acids from acid extract of oil S2.

alternatively aldehyde functionalities. The C=O stretch signals at 1734 (esters) and 1705 cm^{-1} (ketones, acids) have high intensities, in some cases the highest in the spectrum. The aromatic C=C stretch signal at around 1596 cm^{-1} is absent, while a weaker signal at 1603 cm^{-1} (possibly from amino groups, but no certain assignment) is observed instead. The wide ether and/or phenoxy signal at 1300–1150 cm^{-1} is weaker relative to the maximum intensity peaks, and the aliphatic C–H bend at 750 cm^{-1} is weak or not present. The non-biodegraded oils are more similar to phenols, with a wide –OH or N-H hump above 3300 cm^{-1}, and signals at the aromatic C–H and C–C bands. The observations for all samples are summarised in Table 5.

Briefly summarised, the biodegraded oils have more carboxylic acid functionalities and saturated hydrocarbon structures, while the non-biodegraded oils are more phenolic in character.

3.6. Molecular analysis by GC-MS

For identification of individual components of a mixture by GC-MS, a reasonably good separation into individual peaks is necessary. For polar compounds to be run at the conditions generally used for organic geochemical GC-MS analysis, the polar functional groups have to be converted to more volatile products. Using the established procedures of derivatisation with diazomethane, carboxylic acids should give methyl esters, and phenols should give methyl-aryl ethers (in somewhat lower yields).

Fig. 4 shows chromatograms of two acid extracts from oils B2a and S2. In the total ion chromatogram (TIC) of the biodegraded B2a oil shown as the top trace in Fig. 4a, there is nearly no fine structure, only the unresolved complex envelope (UCM) similar to biodegraded oil hydrocarbon chromatograms. Use of selected ion monitoring (SIM) of fragments that represent specific structural units does not yield much additional information. The fragment at $m/e = 57$ represents $C_4H_7^+$ alkyl fragments, which are present as an UCM at quite high intensity. The $m/e = 91$ fragment corresponds to the benzyl cation, and gives a high-intensity UCM. The $m/e = 74$ fragment represents carboxylic acid groups attached to an unsubstituted methylene unit and is strong for *n*-alkanoic acids, but it is of low intensity in this sample. Overall, no molecular information can be obtained from the GC-MS data.

Comparison with the extract from the non-biodegraded oil, Fig. 4b, emphasises the differences. Though still complex, the TIC shows separation into individual peaks. The intensity of the $m/e = 57$ and 74 traces are low, indicating that the alkyl chain content is limited, i.e. a low alkyl acid content. The intensity of the $m/e = 91$ trace is strong, again suggesting a more aromatic and phenolic composition for the non-biodegraded acid fractions.

4. Discussion

4.1. Acidity of biodegraded oils

This work confirms the previous observations of high acidity measured as TAN in biodegraded oils. However as shown in Table 1, no proportional relationship between the level of biodegradation and the acidity is seen in the data set presented here. The total base content also increases with biodegradation, and there is a quite good correlation between the TAN and TBN (correlation coefficient $R^2 = 0.75$, Table 2). This suggests that acid-base equilibria may be important for the observed acidity of the oil. However, identification of the specific functional groups that contribute to the acidity is needed to evaluate the importance of the conjugate acids and bases for the measured acidity and basicity of the oils.

4.2. Composition of the acid fraction

The yields of extractable acids by weight also increases in biodegraded oils (correlation coefficient with TAN: $R^2 = 0.80$, Table 2). The bulk composition of the extracted acids is significantly different between the two classes of oil, with a much lower equivalent weight of the acids for the biodegraded oils (Table 4, Fig. 2). The acidity is distributed in both the asphaltene and maltene fraction of the oil (Table 3). As measured by GPC, the molecular weight of the acids extracted from biodegraded oils is somewhat lower than for the non-biodegraded acid fractions. The infrared spectra show clear distinctions between the two groups (Table 4, Fig. 3), with the biodegradation resulting in more carboxylic and more saturated structures than the original petroleum acids, giving a similar relationship as observed in the Watson et al. (2002) incubation experiments.

To summarise the information from the different methods of characterisation: the average acid in a biodegraded oil is multifunctional, has an equivalent weight in the range 100–>1000 g/mol with a major part in the 300–500 g/mol range, which corresponds quite well with the mass spectrometric analyses reported by Quian et al. (2001) and Tomczyk et al. (2001). The acidic compounds are not amenable to the standard organic chemical analysis techniques for GC-MS, and further molecular information must be found by other techniques.

4.3. Stability and reproducibility

The interpretation of the data from the different analytical procedures is complicated by the apparent instability of the petroleum acids. Both TAN values and

especially extract yields change over time. The TAN values mostly seem to increase slightly over time in repeated determinations, while the extract yield shows a tendency to rise significantly (see sample pairs marked (1) and (2) in Table 1). This may be caused by reactions that increase the content of acidic compounds with high molecular weights or produce higher molecular weight compounds that still are extractable by ethanolic base, possibly initiated by contact with the air. Fresh samples and good sample handling procedures are thus necessary for reliable analysis.

The extraction procedures pose a special problem in this context, since the sum of the TAN values determined for the fractions are low compared to the value determined for initial oil (see Table 3). The cause of this is presently unknown, though reactions that convert acidic groups like carboxylic acids and phenols to non-acid products like esters and ethers can be envisioned, especially since the oil is in contact with strong acids and bases during the extraction procedure. It is not possible to be certain whether the products of such reactions would become part of the extracts or would remain in the bulk oil, since this would depend on which stage of the extraction procedure the reactions occur. Thus, the results based on analysis of the extracts must be used with caution, and there is a clear need for better procedures for extraction. Recoveries from alternative procedures, like the solid phase extraction used by Quian et al. (2001), need to be investigated to determine if such procedures conserve the initial properties of the acidic compounds throughout the procedure.

4.4. Origin of acids in biodegraded oils

The differences observed in the infrared spectra of the extracts and in the GC-MS chromatograms strongly suggest that the acids in biodegraded oil are produced by the microbial degradation processes. A concentration effect resulting from the removal of light hydrocarbons would leave the compositional pattern of the acids largely unchanged. Microbial biomass components seems a probable source for the acidic compounds, as also suggested by Tomczyk et al. (2001), but this still needs to be investigated and clarified.

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