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Pressurized liquid extraction of selected molecular biomarkers in deep sea sediments used as proxies in paleoceanography

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Abstract

Pressurized liquid extraction has been performed on a suite of deep-sea sediments to assess its capability as an extraction technique in the analysis of molecular biomarkers used in paleoceanography. Specific compounds assessed comprise long-chain alkenones, *n*-alkanes, *n*-alcohols and, additionally, one diol and one keto-ol. These have been extracted by both pressurized liquid extraction and ultrasonication for comparison. One key result is that the $U^{K'}_{37}$ index (based on the degree of unsaturation of the alkenones and used as a paleothermometer in paleoceanography) remains intact after both extraction techniques. In terms of biomarker concentrations, which are often used to qualitatively assess changes in marine productivity and/or terrigenous inputs, pressurized liquid extraction is substantially more efficient than ultrasonication, providing higher amounts of extracted constituents, particularly for polar compounds.

Keywords: Pressurized liquid extraction; Molecular biomarkers; Deep sea sediments; $U^{K'}_{37}$ index; Alkenones

1. Introduction

Over the last 15 years, analysis of specific molecular biomarkers in deep sea sediments has become an invaluable technique in paleoceanographic studies, with the data being used to reconstruct paleoclimates over the last 1 million years (see review by Brassell [1]). The resulting knowledge of the global climatic system has relevance to predictions of future global warming, an issue with ecological and socio-economic implications on a planetary scale [2].

In particular, relevant information has been gathered by means of sedimentary analysis of C_{37} alkenones, compounds specifically synthesized by phytoplanktonic Haptophyta algae [1]. Their degree of unsaturation forms the basis of a well established paleothermometer for marine waters, the $U^{K'}_{37}$ index [3]. Their abundances are taken as qualitative indicators of paleo-marine productivity of this algal precursor (e.g. Refs. [4,5]). Other compounds of interest encountered in deep sea sediments comprise land derived long chain *n*-alkanes and *n*-alcohols sourced from higher plant epicuticular waxes, which can provide information on paleo-aridity, wind intensity, and/or riverine runoff (e.g. [6–8]). The capability and potential of these molecular biomarkers as recorders of paleo-environment has led to the rapid expansion of scientific groups interested in

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the organic molecular characterization of deep sea sediments.

Such paleoclimatic studies often involve a large number of samples (e.g. 200–500 samples per core) in order to obtain meaningful paleoclimatic information. This requirement prompts the development of rapid and straightforward analytical methodologies. One of the critical steps is the extraction of organic matter from the sediments, which is most often performed by ultrasonication (e.g. [9–11]), although some groups have reported the use of Soxhlet [12,13] and a flow-blending technique [14]. Most recently, pressurized liquid extraction (PLE) has been adopted by three research groups [12,15,16]. This technique, which extracts biomarkers under high pressure and temperature [17,18], offers a range of advantages over traditional methods including more rapid sample extraction, increased automation and lower solvent consumption and exposure. These factors make PLE very attractive to paleoceanographers and, in the future, this technique could certainly become a standard extraction process in this field.

This study is a comparison between ultrasonication and PLE for the extraction of organic compounds from a variety of deep sea sediments. We have focused on the analysis of the molecular biomarkers commonly used in paleoceanography, namely alkenones, *n*-alkanes and *n*-alcohols. The main goal is to verify whether paleotemperatures derived from the unsaturation pattern of alkenones (the $U^{K'}_{37}$ index) are comparable using these two different extraction methods. Sedimentary abundances of these biomarkers and other qualitative ratios derived from them are also evaluated to check for any dependence on the extraction method.

2. Experimental section

2.1. Materials

HPLC–GC grade dichloromethane and methanol were obtained from BDH (Poole, UK) and *n*-hexane and toluene were from Merck (Darmstadt, Germany). *n*-Hexatriacontane from PolyScience Corporation (Niles, IL, USA) was used as an internal standard.

The sediment samples analyzed in this study were selected from three marine cores from north-western Australia (GC10, GC21 and MUC1) and a marine core retrieved south of Tasmania, (MD972106; two different mixtures, see Table 1 for exact locations). All samples were previously freeze–dried and then manually ground before extraction. At this stage, replicates of each sample were split and analyzed by the two different extraction techniques: ultrasonication and PLE.

2.2. Ultrasonication

As described by Villanueva et al. [11], after the addition of 20 μl of an internal standard (58 ppm *n*-hexatriacontane in toluene), between 2.5 and 5.5 g of dry sediment were extracted, which was repeated three times with dichloromethane in an ultrasonic bath (20 min×3). The organic extracts were combined (~40 ml) and evaporated to dryness under a gentle nitrogen stream.

2.3. Pressurized liquid extraction

Equivalent amounts of sediment were loaded into 11 ml volume stainless steel extraction cells of a

Table 1
Location and identification of sediment samples analyzed in this study

Sample number	Sediment core	Latitude	Longitude
1	MD972106 mixture 1	45°15′S	146°29′E
2	MD972106 mixture 2	45°15′S	146°29′E
3	GC10	18°09′S	116°01′E
4	MUC1	12°00′S	127°50′E
5	GC21	14°49′S	114°16′E

Dionex ASE 200 Accelerated Solvent Extractor (Dionex, Sunnyvale, CA, USA). As with the ultrasonication method, 20 μl of the same internal standard were added to the sediment before extraction.

Extractions were carried out at 1000 p.s.i. and 100 °C (see Section 3.1 for optimization of PLE) as follows: (1) Preheating of the cell to the selected temperature for 2 min and pumping of dichloromethane into the cell; (2) pressurization of the cell to 1000 p.s.i. with 5 min thermal equilibration; (3) static extraction for 2 min; (4) flushing of the extract from the sample into the collection vials; and finally (5) purging of the solvent residue with pressurized nitrogen. Steps 3 and 4 were repeated five times for each extraction cell with the introduction of fresh solvent (about 25% of the total cell volume) after each static phase. The solvent was then combined in one collection vial. The extracts (~25 ml) were then evaporated to dryness under a nitrogen stream.

The evaporated extracts from ultrasonication and PLE were hydrolyzed overnight with 6% potassium hydroxide in methanol at room temperature, in order to eliminate wax esters. The neutral fraction was obtained after back extraction with *n*-hexane three times. Before evaporating, the *n*-hexane extracts were washed with 1 ml of Milli-Q water to remove any KOH residue. The evaporated extracts were transferred to gas chromatography vials and derivatized with bis(trimethylsilyl)trifluoroacetamide (BSTFA+1% trimethylchlorosilane as a catalyst; Alltech, Deerfield, IL, USA) for 1 h at 65 °C, converting all alcohols to trimethylsilyl ethers. The derivatized extracts were then evaporated under a nitrogen stream and redissolved in 50 μl of toluene for injection on a gas chromatograph.

2.4. Gas chromatography

The instrument used was a Hewlett-Packard HP6890 with a flame ionization detector (FID) and an on-column injector. The capillary column was a CP-Sil 5 CB (50 m, 0.25 mm I.D. and 0.25 μm film thickness; Chrompack, Middelburg, Netherlands) and hydrogen was used as carrier gas at a constant pressure of 50 p.s.i. The oven was programmed from 90 °C (holding time of 1 min) to 160 °C at 15 °C/min, 160–280 °C at 10 °C/min with 30 min hold at 280 °C and finally, from 280 to 315 °C at 6 °C/min with a holding time of 6 min. The on-column injector was programmed from 90 °C (holding time of 1 min) to 320 °C at 200 °C/min. The detector temperature was 320 °C. Compound concentrations (expressed as ng/g of dry weight sediment) were derived from gas chromatography (GC)-FID signals relative to that of the *n*-hexatriacontane internal standard.

Selected samples were analyzed by GC–MS for compound identification, using a Hewlett-Packard HP5973 MSD attached to an HP6890 GC. Injections were made using an on-column injector and the same capillary column. Helium was used as carrier gas at a constant pressure of 50 p.s.i. The oven temperature program was from 90 °C (holding time of 5 min) to 160 °C at 20 °C/min, from 160 to 280 °C at 8 °C/min with 55 min hold at 280 °C and finally, from 280 to 315 °C at 10 °C/min with a holding time of 5 min. The mass spectrometer was operated at 70 eV in full scan mode from 50 to 600 m/z.

3. Results and discussion

3.1. Optimization of PLE for molecular biomarkers extraction from sediments

Prior to the comparison of the extraction efficiency of PLE and ultrasonication, the optimum experimental PLE conditions for maximum extraction of alkenones, *n*-alkanes and *n*-alcohols were established (see Sections 3.3 and 3.4 for description of these compounds). Several extractions were performed at different temperatures and pressures (Table 2), always using the same amount (2 g) of Sample 1 (Table 1).

The recoveries obtained for each group of compounds are not significantly affected by the different temperatures and pressures applied (Table 2). This was shown by a series of experiments on Sample 1 at a constant pressure of 1000 p.s.i. and increasing temperatures of 75, 100, 125 and 150 °C. The concentrations of each group of compounds were not affected by changing the extraction temperature, in the 75–150 °C range (Table 2), although this parameter has proven to be an important factor contributing to the increased recoveries of other types of com-

Table 2
Optimization of Pressurized Liquid Extraction for molecular biomarkers extraction from sediment Sample 1

	$P=1000$ p.s.i. $T=75$ °C	$P=1000$ p.s.i. $T=100$ °C	$P=1000$ p.s.i. $T=125$ °C	$P=1000$ p.s.i. $T=150$ °C	$P=1500$ p.s.i. $T=100$ °C	$P=2000$ p.s.i. $T=100$ °C
n-alkanes (ng/g)	1160	1240	1280	1430	1300	1240
n-alcohols (ng/g)	1230	1050	1020	990	1080	1110
Alkenones (ng/g)	3150	3330	2995	3415	3260	3200
$U^{K'}_{37}$-SST (°C)	10.41	10.25	10.40	10.45	10.35	10.16

pounds (see review by Björklund [19]). Since temperature was not a key factor in obtaining high recoveries, a compromise temperature of 100 °C was chosen to carry out tests at different pressures. This lower temperature was selected to avoid any potential for alteration products generated at higher temperatures. The concentrations obtained at three different pressures of 1000, 1500 and 2000 p.s.i. were also very similar for all the compounds, irrespective of the pressure applied (Table 2). This finding is consistent with several previous reports [19]. It appears that the only requirement is that the solvent remains in liquid state, preventing boiling during extraction [19]. Based on these results, a pressure of 1000 p.s.i. and a temperature of 100 °C were selected as suitable, since there is no need to apply greater temperatures or pressures.

One important result from this series of experiments is that the $U^{K'}_{37}$ index (see Section 3.3 for definition) and thus, the inferred paleotemperatures, are not altered by either temperature or pressure (Table 2). These experiments ($n=6$) give a mean estimated temperature value of 10.34 ± 0.11 °C. Therefore, even if other pressure and temperature settings are used (in the 1000–2000 p.s.i. and 75–150 °C ranges), consistent data is obtained. This aspect is important, since other paleoceanographic research groups already using PLE have reported different extraction settings (e.g. 120 °C and 1451 p.s.i. in Ref. [15]; 150 °C, 2000 p.s.i. in Refs. [12,16]).

3.2. Distribution of studied molecular biomarkers

Samples 2, 3, 4 and 5 were selected to perform comparisons between PLE and the ultrasonic extraction methods. Compounds selected for this comparison were long-chain alkenones, long-chain *n*-alkanes and long-chain *n*-alcohols, which are the most frequently used molecular biomarkers in paleoceanographic studies. Concentrations obtained for each sample are shown in Table 3. The distribution pattern of long-chain *n*-alkanes was very similar for the four samples, with a clear predominance of odd numbered *n*-alkanes known to be derived from higher plant epicuticular waxes ([20]; Fig. 1). Note that some compounds were not always quantified in all the samples, due to coelutions and/or low abundances. A coeluting peak identified by GC–MS as a mixture of C_{30} 1,15-diol + C_{30} 15-keto-1-ol has also been quantified.

3.3. $U^{K'}_{37}$ index paleotemperature estimations and alkenone concentrations

The long-chain (*n*-C_{37}–C_{39}) alkenones are di- and tri-unsaturated methyl ketones synthesized by some Haptophyta algae and are characteristic biomarkers in sediments from all oceans [1]. In particular, those with 37 carbon atoms, namely, heptatriaconta-(15E, 22E)-dien-2-one ($C_{37:2}$) and heptatriaconta-(8E, 15E, 22E)-trien-2-one ($C_{37:3}$) have become important for paleoceanographers because of the close relationship between their relative abundances and the temperature of the waters where they were biosynthesized [3]. This correlation is expressed as the $U^{K'}_{37}$ index ($U^{K'}_{37}=C_{37:2}/(C_{37:2}+C_{37:3})$) and has been successfully used as a paleothermometer in oceans all over the world (e.g. [10,21–24]). The most common calibration used to translate $U^{K'}_{37}$ values into SST estimations is that from Müller et al. [27] ($U^{K'}_{37}=$

Table 3
Comparison of the concentrations (ng/g) of the different compounds obtained by ultrasonication (US) and PLE

Compound	Sample 2[a]		Sample 3[b]		Sample 4[c]		Sample 5[c]	
	PLE	US	PLE	US	PLE	US	PLE	US
C25	124	120	88	73			30	39
C26	57	110	52	68	28	37	21	40
C27	204	200	183	133			62	64
C28	65	111	53	72			29	49
C29	510	350	326	193			103	94
C30	93	99	72	66			30	39
C31	870	640	650	372	223	130	273	177
C32			63	51			34	32
C33			529	301			262	169
C35			73	51			52	29
Σ *n*-alkanes	1900±100	1620±170	2087	1379	250±20	167±6	900±50	730±30
CPI[d]	5.9	3.1	5.9	3.3			5.1	2.7
C24-ol	460	300	367	242	252	94	29	22
C26-ol	500	350	272	141	62	28	37	31
Σ *n*-alcohols	960±180	650±70	639	383	314±12	122±13	66±5	53±5
Diol + ketol	350±80	160±50	1726	191	460±30	21±7	77±17	18±5
$C_{37:4}$	118	82	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
$C_{37:3}$	1370	930	38	11	n.d.	n.d.	n.d.	n.d.
$C_{37:2}$	879	590	1719	655	n.d.	n.d.		
Σ alkenones	2370±50	1600±170	1757	666				
$U_{37}^{K'}$[e]	0.391	0.389	0.978	0.983				
SST (°C)[f]	10.51±0.04	10.45±0.2	28.32	28.45				

n.d., not detected.
Empty cells correspond to non quantifiable values due to coelution or low abundances.
[a] Average of three replicates.
[b] No replicates.
[c] Average of two replicates.
[d] Sample 2: CPI = 3×(C25 + C27 + C29 + C31)/(4×(C26 + C28 + C30)). Samples 3 and 5: CPI = 4×(C25 + C27 + C29 + C31 + C33)/(5×(C26 + C28 + C30 + C32)).
[e] $U_{37}^{K'} = C_{37:2}/(C_{37:2} + C_{37:3})$.
[f] Sea surface temperatures (SST) were calculated after [27]: $U_{37}^{K'} = 0.033*SST + 0.044$.

0.033*SST + 0.044), where a large number of marine core tops ($n = 370$) were compared with the present annual mean temperature of the overlying waters. A large expansion in the practical application of this paleoceanographic tool is evident from the exponential increase in the number of publications per year based on this index [25]. A third alkenone with four unsaturations ($C_{37:4}$), although rarely found, has also been reported in sediments from high latitudes. However, its relationship with sea surface temperature (SST) is still a matter of debate (e.g. [26]).

Due to the expansion in the use of the $U_{37}^{K'}$ index, and considering the advantages of PLE systems, it is likely that the use of this easy new extraction technique will become a standard extraction process in paleoceanography. However, it is necessary to evaluate whether paleotemperatures from PLE extracted alkenones are comparable to previously used extraction methods.

In the present study, three of the samples (1, 2 and 3) contained significant concentrations of alkenones to be quantified. As already mentioned, different temperature and pressure conditions during PLE do not affect the $U_{37}^{K'}$ ratios, supporting the analytical robustness of this proxy over a broad range of possible PLE settings.

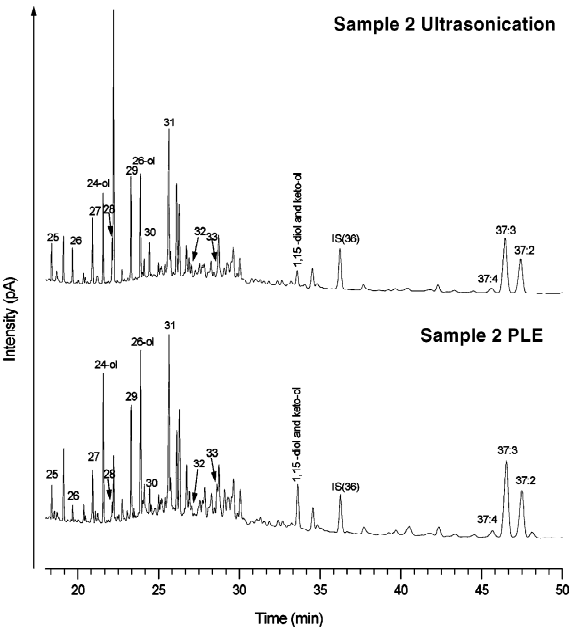

Fig. 1. Comparison of two GC chromatograms of Sample 2 (MD972106 Mixture 2) obtained by the two different extraction methods. *x* refers to long chain *n*-alkanes, *x*-ol to long chain *n*-alcohols and *x*:*y* to C*x* alkenones with *y* unsaturations. I.S.: internal standard, *n*-hexatriacontane.

Comparison of the PLE and ultrasonication methods indicates that the $U^{K'}_{37}$ ratios are also unaffected by the method chosen. Table 3 illustrates that SST calculated from PLE extracted samples (using the equation $U^{K'}_{37} = 0.033*SST + 0.044$; [27]) matches very closely the value obtained from ultrasonication (10.51 ± 0.04 vs. 10.45 ± 0.2 °C, respectively). Therefore, the calculation of $U^{K'}_{37}$ is not affected by the extraction method used in the analysis, which validates the PLE technique and confirms the robustness of the $U^{K'}_{37}$ method.

When considering alkenone abundances, however, this study shows an increased recovery using PLE (Table 3 and Fig. 2). This emphasizes the usefulness and greater efficiency of PLE as an extraction technique, but also indicates that comparing biomarker abundance records obtained using different extraction methods requires caution. However, for each method, results for alkenone extraction always fall within a 10% error (Table 3). This suggests that ultrasonic extraction is still a valuable method, which will provide consistent alkenone values when data sets with the same extraction method are used.

3.4. Long-chain n-alkanes and n-alcohols

Other major organic constituents in the studied samples were the odd C_{25}–C_{33} *n*-alkanes which, together with the even *n*-alcohols (C_{24} and C_{26}), are often used as indicators of terrestrial input to the marine environment (e.g. [6,7]). As shown in Table 3 and Fig. 2, the total amount of these compounds extracted by PLE was consistently higher than was obtained by ultrasonication for all samples. For example, *n*-alkane and *n*-alcohol concentrations were 23 and 36% higher for Sample 2 (average of three replicates), using PLE extraction compared to ultrasonication. Hence, care needs to be taken when comparing data sets of compound concentrations collected using both methods, since it could lead to some paleoenvironmental misinterpretations. However, data sets obtained with the same extraction method have an averaged uncertainty typically below 10% (Table 3). This level of uncertainty is usually enough to distinguish qualitative paleoclimatic trends or changes.

In additon to the improved extraction efficiency of PLE, a different behaviour of odd vs. even *n*-alkane homologous is also observed. Concentrations of odd numbered C_{25}–C_{33} *n*-alkanes were higher using PLE while even numbered C_{26}–C_{32} *n*-alkane abundances were similar for both methods (Table 1). This result is of particular importance when calculating the Carbon Preference Index (CPI; ratio of the amounts of odd-carbon to even-carbon *n*-alkanes), which is often used to distinguish between terrestrial higher plants (CPI values between 3–5 and 10) and petroleum hydrocarbons (CPI = 1; e.g. Ref. [28]). In the comparative work presented here, CPIs obtained ranged from 5.1 to 5.9 for PLE and from 2.7 to 3.3 for ultrasonication (Table 1). These differences could lead to misinterpretations on the actual sources of these biomarkers in the sedimentary record. This discrepancy between extraction methods is difficult to explain, but may result from the different extent to which terrigenous markers are linked to the sedimentary matrix. For instance, the strong interaction between organic compounds and mineral surfaces (e.g. clays), has been recognized as influencing the

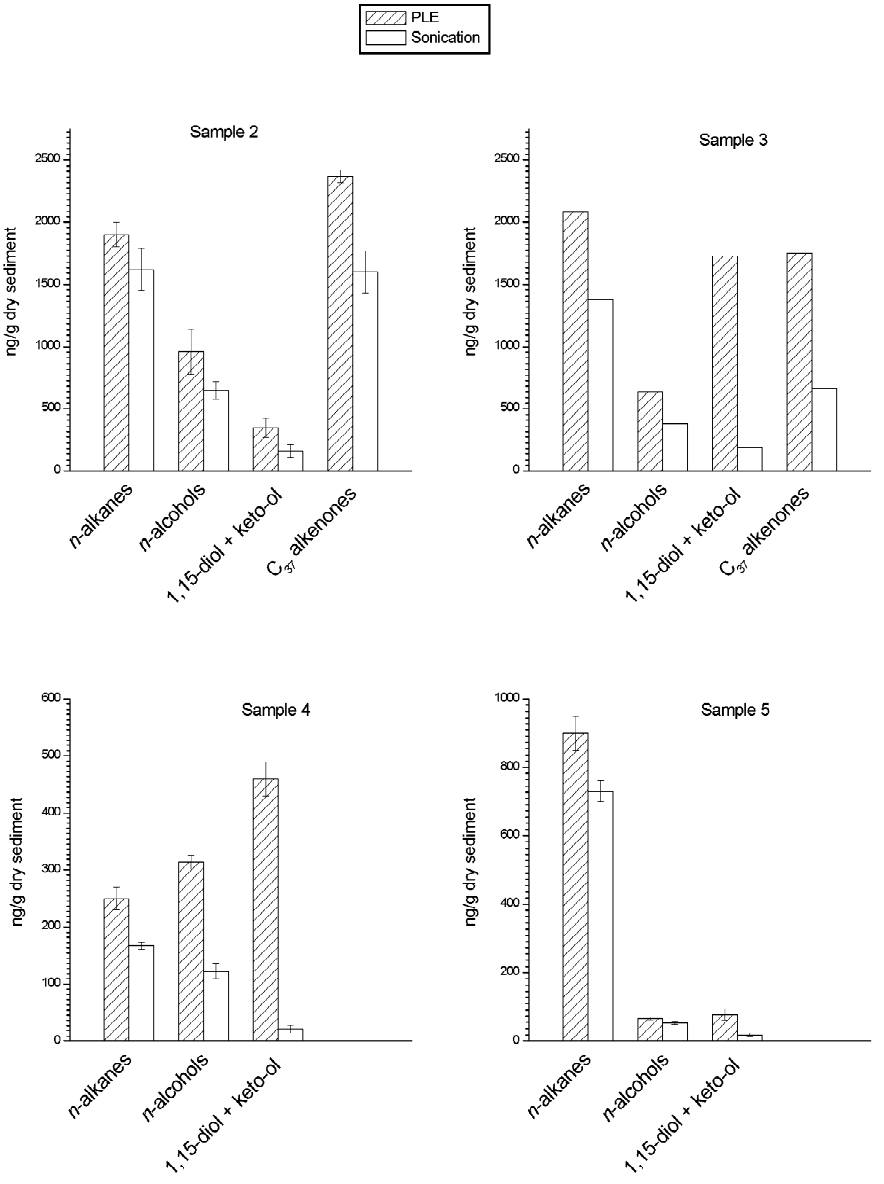

Fig. 2. Comparison of the extraction efficiency of PLE and ultrasonication. Concentrations of the different biomarkers studied in each sample. The abundances obtained with the PLE technique were significantly higher than those from ultrasonication (see text). The standard deviations for each measurement are also displayed.

efficiency of transportation (and preservation) of organic matter between the sea surface and bottom sediments (e.g. Refs. [29,30]). Thus, one possibility to explain why the PLE method leads to a stronger terrestrial higher plant signal could be due to the increased extraction efficiency of this method. Epicuticular wax components bound to clay surfaces in the sediment may be more efficiently removed. If this is the case, the PLE method may more accurately reflect the input of terrestrial material in sediments than ultrasonication extraction. Nevertheless, caution should be used in comparisons of such

indices for data sets collected using both extraction methods.

3.5. Additional extracted compounds

Other marine organic compounds quantified in this study were the C_{30} 1,15-diol and C_{30} 15-keto-1-ol, which are often encountered in recent marine sediments (see review by Morris and Brassell [31]). Although their biological source in open oceanic waters is still not clear, they are found in some algae from the class Eustigmatophyceae [32]. In our samples, C_{30} 1,15-diol and C_{30} 15-keto-1-ol were quantified as a single peak since they co-eluted using the chromatographic conditions employed for GC–FID alkenone analysis (Fig. 1). The concentrations of these alcohols obtained by PLE were almost one order of magnitude higher than those obtained by ultrasonication (Table 3). This result suggests that these compounds may be easily missed when extracting with the traditional ultrasonication technique.

4. Conclusions

Molecular biomarker extraction of four marine sediment samples has been performed using two different extraction techniques: PLE and ultrasonication. Comparison of both methods reveals a consistently more efficient extraction of the sedimentary alkenones, long-chain alkanes, alcohols, diols and keto-ols when using PLE. More efficient extraction together with the automation of the process and the decrease in solvent usage indicate that PLE extraction will be a very useful technique in paleoceanographic studies where a large number of samples typically require processing.

The robustness of the alkenone based paleothermometer, the $U^{K'}_{37}$ index, has been confirmed. The relative abundances of the di- and tri-unsaturated alkenones are not affected by the extraction technique used and, therefore, the $U^{K'}_{37}$-SST estimations are the same for both methods and the respective data sets will be compatible. The same is true when different conditions of temperature (75–150 °C) and pressure (1000–2000 p.s.i.) are used to extract the samples with PLE. One difference was the greater extraction of odd *n*-alkanes compared with even *n*-alkanes using PLE. This leads to higher CPI values for the samples extracted with PLE possibly due to greater extraction efficiency of epicuticular waxes preferentially bound to clay surfaces.

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