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Application of microwave-assisted extraction to the analysis of biomarker climate proxies in marine sediments

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Abstract

Microwave techniques are widely used in acid digestion of solid samples. Their use in the extraction of organic analytes from environmental samples is less widespread, despite commercial devices being available for this purpose and the potential for reducing analysis time and solvent consumption. We report the use of microwave-assisted extraction (MAE) of biomarkers (chlorins and long-chain C_{37} alkenones), which are used as palaeoclimatic proxies in marine sediments. Factorial design was applied to determine the influence of temperature, volume of solvent and extraction time on the efficiency of the extraction of total chlorins. We found that only changes in temperature produced significant variation in yield. The extraction temperature of MAE was then optimised for both chlorins and alkenones. Equivalent results to repeated extractions by ultrasonication were obtained from a single extraction step of 5 min using 10 ml of solvent at a temperature of 70 °C. MAE was found to be a more efficient, faster and less labour-intensive method than ultrasonic extraction. Assessment of the influence of extraction conditions for MAE on the relative recovery of alkenones showed that this technique is a feasible option in the measurement of $U^{K'}_{37}$ in marine sediments.

1. Introduction

Microwave assisted extraction (MAE) is based on the direct application of electromagnetic radiation to a material (e.g. organic solvent, plant tissue) which has the ability to absorb electromagnetic energy (microwaves) and to transform it into heat. Unlike conventional heating by infrared energy or thermal conductivity, the increase in temperature occurs simultaneously in the whole volume of solvent. This process is caused by the multiple collisions of the solvent molecules as they realign in the oscillating electromagnetic field, generating energy in the form of heat (Letellier and Budzinski, 1999). Compared with conventional methods, such as ultrasonic extraction and Soxhlet extraction, the advantages of MAE are reported to be a higher recovery of the analyte, shorter extraction times and the use of smaller quantities of solvent (e.g. Pastor et al., 1997; Tomaniová et al., 1998; Blanco et. al., 2000; Jayaraman et al., 2001).

MAE can be performed in open or closed vessels (see review in LeBlanc, 1999). In open systems, the extraction occurs at atmospheric pressure and with variable energy input. In closed systems, extraction takes place at controlled pressure (up to 5 atm) and a temperature that may exceed the boiling point of the solvent under atmospheric conditions, to increase extraction efficiency.

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In commercially-available closed systems, a large number of samples can be processed simultaneously.

Since the introduction of MAE of organic compounds by Ganzler et al. (1986), the application of open systems has been reported for the extraction of a wide range of components, including polyaromatic hydrocarbons (PAHs), phenols, total petroleum hydrocarbons, pesticides, polychlorinated biphenyls (PCBs), triazines and fats from a variety of matrices, e.g. soils, sediments and biological tissues (see Letellier and Budzinski, 1999; Letellier et al. 1999; Camel, 2000; Eskilsson and Björklund, 2000; Kaufmann and Christen, 2002 and references therein). Nonetheless, to our knowledge, MAE in closed vessels has not been previously employed in biomarker studies.

Here, we present and appraise the optimisation of MAE of biomarkers belonging to two distinct compound classes, namely chlorins and long-chain C_{37} alkenones, used as palaeoclimate proxies in marine sediments. Sedimentary abundance of chlorins can be related to past changes in primary productivity in the depositional environments (e.g. Rosell-Melé, 1994; Summerhayes et al., 1995; Harris et al., 1996). For example, the presence of certain bacteriochlorophylls or their diagenetic counterparts ($>C_{33}$ porphyrins, e.g. Repeta et al., 1989) can be used to reconstruct the occurrence of anoxygenic photosynthesis. Alkenones in sediments are used to calculate the $U^{K'}_{37}$ index, a proxy for past sea surface temperatures (Brassell et al., 1986; Prahl and Wakeham, 1987), which is calculated from the relative abundance of $C_{37:2}$ [heptatriaconta-(15E,22E)-dien-2-one] and $C_{37:3}$ [heptatriaconta-(8E,15E,22E)-trien-2-one] unsaturated ketones. Reconstruction of sea surface temperatures is based on the linear relationship between $U^{K'}_{37}$ and temperature (Prahl and Wakeham, 1987; Müller et al., 1998).

In order to produce a detailed and meaningful palaeoclimatic record, it is necessary to process a large number of samples usually characterised by small size ($\sim$1 g) and low organic matter content. Ultrasonic extraction is often the preferred method of sample preparation in palaeoclimate studies (e.g. for the alkenones, see Rosell-Melé et al., 2001). It is a relatively inexpensive procedure, although arguably, both time- and labour-consuming since repeated extraction of the sediment is required to extract the analytes with a recovery close to 99%. A fast alternative is accelerated solvent extraction (ASE), based on applying high temperature and pressure to the sample in a relatively low amount of solvent. Commercial systems are expensive and completely dedicated to the operation. As an alternative, we have examined MAE, which is widely employed in the acid digestion of sediments and minerals, and the commercial options are cheaper than ASE while allowing the simultaneous extraction of multiple samples. The tests have been performed in a commercially available device, and the results are compared with those from the extraction of the same samples using an ultrasonic bath.

2. Experimental

2.1. Ultrasonic extraction

Approximately 1 g of freeze-dried and homogenised sediment was extracted using 4 ml of dichloromethane/methanol mixture (3:1) in an ultrasonic bath (Decon Lab Ltd.). Each sample was extracted three times for 15 min. The supernatant was separated from the sediment by centrifuging at 3000 rpm for 5 min in a Mistral 2000 centrifuge (MSE) and then decanted. The solvent from the combined supernatant was removed using a CentriVap Vacuum Concentrator (Labconco).

2.2. MAE

As in the ultrasonic method, $\sim$1 g of freeze-dried sediment was extracted with dichloromethane/methanol (3:1). This solvent mixture is chosen because it efficiently absorbs microwave energy and converts it into heat (Letellier and Budzinski, 1999), and it is the solvent mixture used conventionally in ultrasonic extraction. The apparatus employed was a MARS 5 microwave accelerated reaction system (CEM) equipped with Greenchem pressure vessels with 100 ml Teflon liners. The device allows the simulataneous processing of up to 14 samples (maximum 20 g of sediment in each extraction vessel) at controlled temperatures of up to 200 °C with magnetic stirring. After extraction, the vessels were left to cool to a temperature below 30 °C ($\sim$20 min at room temperature), and the extraction mixture was transferred into 15 ml Pyrex tubes for separation and removal of solvent as in the ultrasonication procedure.

2.3. Quantification of biomarkers

For total chlorin analysis, sample extracts were redissolved in 1 ml of acetone and analysed by visible spectrophotometry using a photodiode array detector coupled to a quaternary pump (Dionex), with methanol (HPLC grade, Fisher) as mobile phase. Relative total chlorin content was estimated by measuring the absorbance of the extracts in the Soret band (410 nm) (Rosell-Melé, 1994). Peak areas corresponding to samples extracted under different conditions, normalised to 1 g dry sediment, were then compared. After silylation of the total extract with bis-(trimethylsilyl)-trifluoroacetamide (BSTFA; Sigma Aldrich), alkenone abundances were determined by gas chromatography with a flame ionization detector (Varian GC 3800) with split injection using an HP1 fused silica capillary column (60 m$\times$0.32 mm internal diameter) with hydrogen as

carrier gas (12 psi). The oven temperature was programmed at 50–140 °C at 10 °C/min, 140–310 °C at 6 °C/min and at 310 °C for 35 min. For quantification, n-hexatriacontane (Sigma Aldrich) was used as the internal standard and added to the sediment prior to extraction.

3. Results and discussion

3.1. Influence of key parameters

Previous studies have established that the efficiency of the recovery of the analyte depends on the extraction conditions, although there is no complete agreement on the significance of the various potentially key factors such as temperature, volume of solvent and extraction time. Most studies show increase in extraction efficiency with increase in temperature due to improved desorption of the analytes from the matrix and higher analyte solubility (e.g. Lopez-Avila et al., 1995; Chee et al., 1996; Llompart et al., 1997). Also, at higher temperatures, solvent viscosity and surface tension decrease, facilitating penetration of the matrix (e.g. sediment). Other researchers, however, report no significant influence of temperature on recovery (e.g. Barnabas et al., 1995; Silgoner et al., 1998). Reduced yields have been reported in some cases, probably due to degradation of thermolabile compounds (e.g. Lopez-Avila, 1996; Font et al., 1998). Most researchers have found that a change in the volume of solvent employed does not lead to a significant variation in the recovery of analytes (e.g. Barnabas et al., 1995; Hasty and Revetz, 1995). A few studies, however, did observe that the amount of solvent employed varied with extraction efficiency. For instance, Chee et al. (1996) reported decreased recovery of PAHs with an increase in solvent volume. A common observation, however, is that it is necessary for the whole sample to be immersed in solvent to avoid electrical arcing (Barnabas et al., 1995). Finally, extraction time (time interval after the extraction temperature is reached) of 5 min or less is often reported as sufficient for an extraction efficiency similar to or higher than that achieved using traditional methods (e.g. Chee et al., 1996; Carro et al., 1999). Others, however, found it necessary to employ longer extraction times to obtain maximum recovery of analytes (e.g. Font et al., 1998; Molins et al., 1997; Silgoner et al., 1998).

The significance of the potentially three key factors (temperature, volume of solvent and extraction time) identified by previous workers and the interaction between them were studied in a screening factorial design. To simplify the procedure, only yields of chlorins were analysed in this part of the study. A set of eight experiments was carried out with each factor at two levels, usually known as 'high' and 'low' (Tables 1 and 2; Miller and Miller, 1993, p. 182). The conditions chosen for MAE were as similar as possible to those for ultrasonic extraction to facilitate comparison between both methods. The temperatures employed were lower than those reported by previous workers in order to shorten cooling times and prevent possible decomposition of some of the analytes at higher temperatures. To evaluate the precision of the method, each experiment was performed in triplicate.

Table 1
Factor levels for screening factorial design

Factor (variable)	Low (−)	High (+)
Volume of solvent (v), ml	4	16
Temperature (T), °C	40	80
Extraction time (t), min	5	15

Table 2
Design matrix and response values in factorial design[a].

Run No.		Temperature (T)	Volume of solvent (v)	Extraction time (t)	Relative yield of chlorins (%)	RSD (%) $n=3$
1	−	−	−	−	73	4.4
2	t	−	−	+	74	1.5
3	v	−	+	−	81	2.4
4	tv	−	+	+	65	2.0
5	T	+	−	−	91	4.5
6	tT	+	−	+	89	3.6
7	vT	+	+	−	100	4.0
8	vtT	+	+	+	97	1.0
9	US×3				90	3.1
10	US×1				59	2.1

[a] Chlorin yield is normalised to the highest value (80°C, 16 ml solvent and 5 min holding time). US×3 − ultrasonic extraction (three extractions) and USx1− ultrasonic extraction (1 extraction). RSD—relative standard deviation, "−" indicates low factor, "+" indicates high factor.

The results of the significance study are shown in Table 2 and Fig. 1 (note that the that relative standard deviation, RSD, did not exceed 4.5%). The effect of each individual factor was assessed by calculating the average difference in response (recovery of chlorins) between the experiments when the levels of the other factors remained fixed. This and the interaction between each pair of factors (e.g. temperature plus volume, volume plus time, etc. — first order interactions) and between all three factors (second order interaction) were calculated according to the procedure outlined in Miller and Miller (1993, p. 181). An analysis of variance (ANOVA) was carried out to test the statistical significance of each factor and interaction.

In this design, only the effect of temperature appears to be significant (Fig. 2). Therefore, only this parameter was optimised to maximise extraction efficiency. It was also evident that MAE at 80 °C produced chlorin recoveries comparable to those obtained by ultrasonication (Table 2 and Fig. 1). In addition, when using MAE, it was found to be sufficient to extract each sample only once to obtain yields equivalent to those obtained using ultrasonication with three extractions (Table 2 and Fig. 1). It is apparent that our results show that MAE is a faster and less labour-consuming method than ultrasonication. Arguably, the higher temperature used in MAE may lead to alteration in the original composition of individual chlorins in the sediment. However, the wavelength of 410 nm used for measurement of chlorin abundance here and in other palaeoclimate investigations only accounts for the presence of the ring structure of the chlorins, not the substituents

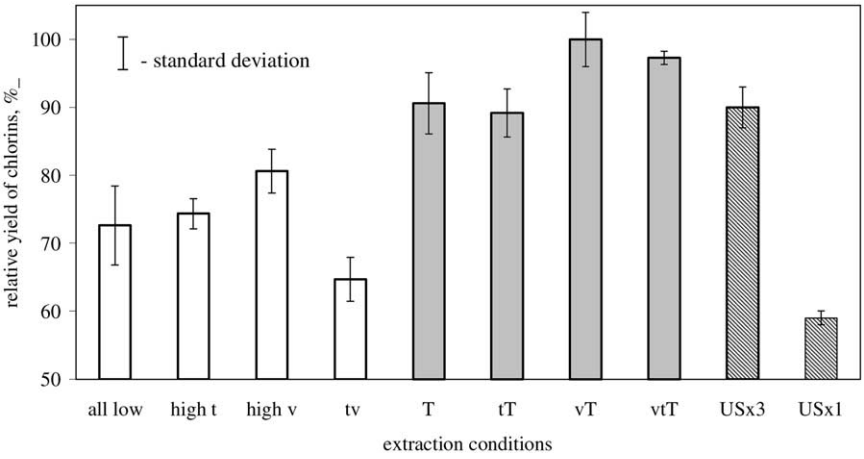

Fig. 1. Average relative yield of pigments (and standard deviation) in screening factorial design normalised to the highest value (at 80 °C, 16 ml solvent and 5 min extraction time), at low temperature (empty bars), at high temperature (shaded bars), and using ultrasonic extraction (patterned bars). Abbreviations stand for: *T*: temperature, *t*: time, *v*: volume of solvent, US×3: ultrasonic extraction repeated three times, US×1: single ultrasonic extraction.

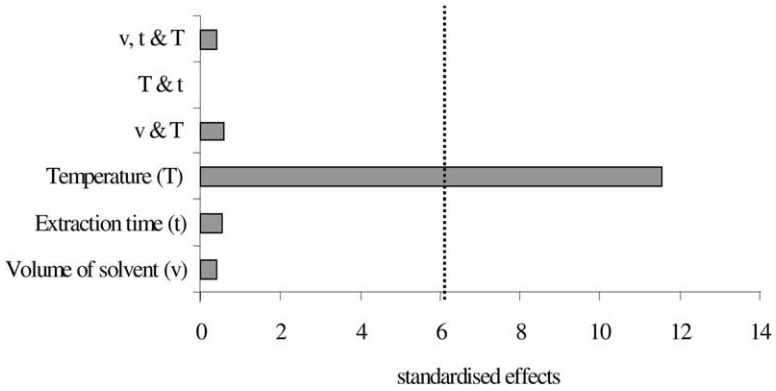

Fig. 2. Representation of the standardised main effects in the factorial design including two- and three-factor interactions. The dotted vertical line indicates the statistical significance bound for the effects – tabulated F_{crit} = 6.115 (at 95% confidence level) (Miller and Miller, 1993, p. 223). Effect higher than F_{crit} is considered significant.

(Jeffrey et. al., 1997). Hence, structural alteration of the chlorins would be irrelevant to these results.

3.2. Optimisation of extraction temperature

A set of seven MAE experiments was conducted at temperatures ranging from 50 to 110 °C using 10 ml of solvent. This volume of solvent is recommended by the manufacturer for the most efficient performance of the temperature probe. An extraction time of 5 min was adopted. The influence of temperature on the yields of total chlorins, di- and tri-unsaturated C_{37} alkenones and their relative concentrations (expressed as $U^{K'}_{37}$; Prahl and Wakeham, 1987) was assessed. Results of MAE were also compared with those obtained by ultrasonication (Table 3 and Fig. 3).

3.2.1. Biomarker yields

MAE at temperatures from 50 to 80 °C produced recoveries of chlorins similar to those obtained using ultrasonication. A further increase in temperature resulted in greater recoveries for MAE. The maximum recovery was achieved at 110 °C (Table 3 and Fig. 3a). For alkenones, however, microwave extraction efficiency in the 50–80 °C interval was significantly higher than that of ultrasonication. Also, alkenone yields rose by 39% when the temperature increased from 50 to 70 °C. Further increase in temperature, however, caused alkenone yields to decrease. One explanation is that at higher temperatures a large part of the dichloromethane remains in gaseous form and does not participate in the extraction. Although gases do not absorb microwave energy and therefore solvent vapour should quickly cool and condense, the large volume of the extraction vessel (100 ml) may allow a significant proportion of the solvent to remain vaporized. This probably has a stronger effect on dichloromethane than on methanol because of the lower boiling point of the former. Chlorins are more soluble in methanol than in dichloromethane because the polarity of methanol is higher. That may explain why an increase in temperature from 70 to 100 °C did not cause a decrease in extraction efficiency of chlorins, while impairing that of alkenones.

A temperature of 70 °C was chosen as the optimal value for the joint MAE of chlorins and alkenones. The rationale behind this is two-fold. The recovery of alkenones is more sensitive to temperature than that of chlorins and it is a maximum at this temperature.

Moreover, the abundance of alkenones in marine sediments is often close to the detection limit because of the small samples available in these studies. In this work, the highest yield of alkenones (315 ng/g) was reached using MAE at 70 °C compared with 188 ng/g for ultrasonic extraction, a 68% increase. In contrast, chlorin yields at this temperature were as high as those for ultrasonication (Table 3 and Fig. 3a). This relatively low temperature (most applications cited in the literature use temperatures higher than 100 °C) also allows a shorter cooling time, which further expedites the processing of samples.

3.2.2. Effects of using MAE in the measurement of $U^{K'}_{36}$

MAE has been reported previously as a method with low selectivity (e.g. Camel, 2000 and references therein). It is not surprising, therefore, that a change in extraction temperature did not influence significantly the relative recovery of $C_{37:2}$ and $C_{37:3}$ alkenones. Comparison of between- and within- experiment standard deviations for $U^{K'}_{37}$ values gave $F(1.98) < F_{crit}(2.46)$ at $\alpha = 0.05$ (Miller and Miller, 1993, p. 60). Comparison between MAE and ultrasonication using a *t*-test (Miller and Miller, 1993, p. 55) showed that both extraction methods were statistically similar ($F(2.25) < F_{crit}(4.46)$ at $\alpha = 0.05$ and $t(0.35) < t_{crit}(2.85)$ at $\alpha = 0.01$). This shows that MAE did not introduce a bias in $U^{K'}_{37}$ determination compared with the traditional ultrasonic method and that MAE can be used as an alternative in alkenone extraction and analysis.

Based on the empirical relationship for estimating sea surface temperature from $U^{K'}_{37}$, an error of 1% in the

Table 3
Recovery of biomarkers using ultrasonic extraction (US×3) and microwave assisted extraction (MAE) at different temperatures, normalised to MAE at 70 °C for alkenones ($C_{37:2}$ and $C_{37:3}$) and at 110 °C for chlorins.

	°C	Yield of alkenones (ng/g dry sediment)	Relative yield of alkenones (%)	RSD (%) $n=3$	Relative yield of chlorins (%)	RSD (%) $n=3$	$U^{K'}_{36}$
US×3		188	60	6.0	80	3.1	0.538 (0.011)
MAE	50 °C	266	72	4.1	81	5.0	0.554 (0.013)
	60 °C	244	77	0.4	78	3.3	0.525 (0.014)
	70 °C	315	100	3.1	83	5.2	0.539 (0.012)
	80 °C	288	91	4.5	81	4.2	0.529 (0.011)
	90 °C	199	69	2.0	93	6.8	0.562 (0.005)
	100 °C	224	71	3.4	97	1.4	0.523 (0.015)
	110 °C	183	58	2.4	100	7.7	0.558 (0.012)

RSD— relative standard deviation. $U^{K'}_{36}$ is expressed as the mean and, in parentheses, standard deviation.

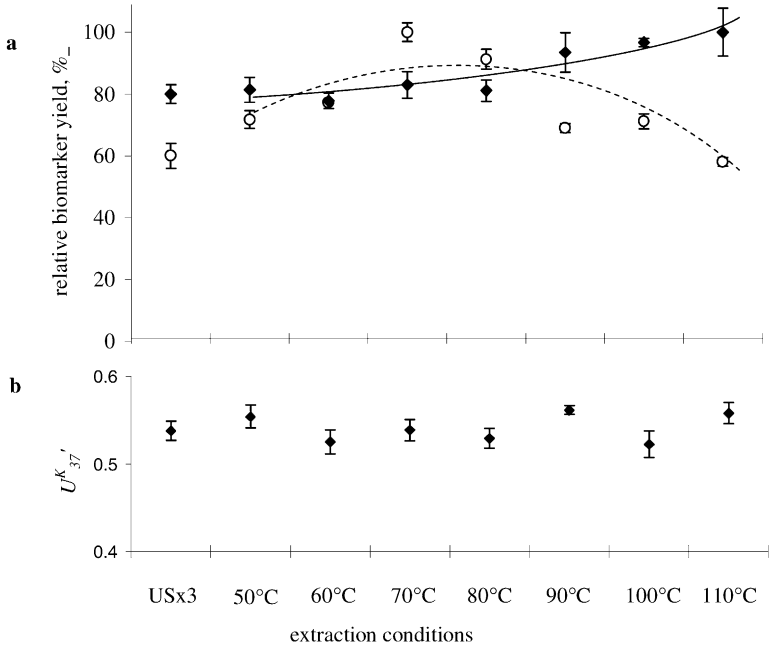

Fig. 3. (a) Relative yield of biomarkers (○ and dashed line - - - $C_{37:2}+C_{37:3}$ alkenones; ◆ and solid line — chlorins) using three rounds of ultrasonic extraction (US×3) and microwave assisted extraction (MAE) at different temperatures. Values are normalised to MAE at 70 °C for alkenones and at 110 °C for pigments. (b) $U^{K'}_{37}$ values obtained at different extraction conditions.

measurement of $U^{K'}_{37}$ translates to an error in the estimate of sea surface temperature of 0.3 °C (Prahl and Wakeham, 1987; Müller et al., 1998). Given that surface ocean temperatures can vary between 1 and several degrees Celsius over a range of timescales of hundreds to thousands of years (e.g. Rosell-Melé et al., 1998), an analytical error of less than 0.5 °C (or lower than 0.0165 in the standard deviation of $U^{K'}_{37}$) may be considered negligible. This study reports a standard deviation in the mean of all MAE experiments of 0.016, over the whole range of temperatures, and an average standard deviation in each experiment of 0.011, which implies that this technique is suitable for future $U^{K'}_{37}$ studies.

4. Conclusions

The use of closed-vessel, microwave-assisted extraction has been appraised for the analysis of biomarkers in marine sediments. Three parameters (temperature, volume of solvent and extraction time) were investigated which, according to previous research, could influence extraction efficiency. Temperature was found to be the only parameter that had a significant influence on yields of the biomarkers. Equivalent values of $U^{K'}_{37}$ were obtained using either MAE or ultrasonication. The use of MAE does not introduce any bias in the measurement of the alkenone paleotemperature proxy. MAE was found to be a faster, less laborious and more efficient extraction procedure than ultrasonication. The technique represents a viable alternative to traditional ultrasonic extraction for the analysis of chlorins and long chain alkenones and, therefore, probably other biomarker lipids in marine sediments.

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