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Impact of saponification and silver-nitrate purification on lacustrine alkenone distributions and alkenone-based indices

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ABSTRACT

A growing interest in lacustrine alkenones as a proxy for continental paleotemperature reconstructions accompanied important methodological improvements over the past two decades. New gas chromatography (GC) columns were used for alkenone analysis, that drastically improved alkenone separation, especially for freshwater lakes. However, these recent advances are sometimes not sufficient in separating compounds that interfere with alkenones in the resulting chromatograms and concurrently, new chemical procedures were implemented to further clean up the samples. Here we investigate the impact of two clean-up procedures, saponification and silver-nitrate purification, on alkenone distribution, alkenone quantification, and C_{37} alkenone-based indices, including the U^K_{37} index. The silver-nitrate purification modified the C_{37} alkenone distribution and thus the C_{37} alkenone-based indices, especially the U^K_{37} index, in 6 out of the 9 studied samples by further retaining alkenones with more double bonds. These changes would result on an average error of 3 °C in reconstructed temperatures. Saponification also influenced the C_{37} alkenone distribution mainly by removing co-eluting compounds, thereby improving the quality of the results. Both saponification and purification resulted in the reduction of the C_{37} alkenone concentration by almost half. Clean-up steps should thus be used carefully, paying particular attention to any change in alkenone distribution and concentration. Limiting the use of additional clean-up steps reduces the risk of modifying the alkenone distribution.

1. Introduction

Alkenones are a group of C_{35} to C_{42} aliphatic unsaturated ketones with 2–4 double bonds which are produced by certain groups of haptophyte algae within the Isochrysidales order [1,2]. Lacustrine alkenones have been successfully applied to reconstruct paleotemperatures in high-latitude lakes, since their degree of unsaturation varies with temperature [e.g., 1,3,4]. As lacustrine alkenones are reported in more and more lakes [e.g., 5], their potential as a proxy for continental paleotemperature reconstructions is growing.

The growing interest in lacustrine alkenones as a proxy was accompanied by improvements in analytical methods and chemical processing to prepare and analyze these compounds. In particular, the introduction of a mid-polarity, poly(trifluoropropylmethylsiloxane) gas chromatography (GC) stationary phase considerably improved lacustrine alkenone analysis [6].

Despite the improved separation achieved by the novel GC columns, lacustrine sediment matrices commonly contain compounds co-eluting with alkenones [e.g., 7]. These co-eluting compounds can modify the alkenone signal and lead to the distortion of reconstructed temperatures [e.g., 8]. Therefore, co-eluting compounds have to be removed. Saponification is widely used to remove wax esters and argentation chromatography, a purification procedure using silver nitrate impregnated silica gel introduced by D'Andrea et al. [9], is commonly used to eliminate the remaining compounds co-eluting with alkenones [e.g., 4,10,11]. More recently, silver thiolate functionalized silica gel has been used as an alternative method for removing co-eluting compounds [e.g. 7,12]. There have not, however, been any systematic evaluations on how these clean-up steps impact alkenone distribution in lacustrine sediments.

The goal of this study is to evaluate the impact that saponification and silver-nitrate purification have on alkenone distributions and quantification as well as on the indices calculated based on alkenones and used for paleotemperature reconstructions.

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2. Material and methods

2.1. Sample preparation

Surface sediments from 9 Swiss freshwater lakes containing Group I alkenones or a mixture of Group I and Group II alkenones, based on alkenone distributions and RIK_{37} values, were used for this study. Sediments (1–5 g) were freeze-dried, ground and homogenized before extraction with an accelerated solvent extraction system ASE350 (Dionex) with dichloromethane (DCM):methanol (MeOH) (9:1, v/v) at 120 °C and 1200 psi. The total lipid extracts (TLEs) were split in two equal parts. One part of the TLE was saponified by heating the extract at 65 °C for 3 h in 1 ml of 1 M KOH in MeOH:H_2O (95:5, v/v). After cooling to room temperature, the mixture was quenched with NaCl (5 %), acidified to pH 2 with concentrated HCl and extracted with hexane three times. A final clean-up was achieved on a silica gel column with DCM (100 %).

The saponified and non-saponified parts of the TLE were separated into alkane, ketone and polar fractions using silica gel flash chromatography with hexane, DCM and MeOH as eluents, respectively.

After GC analysis, the saponified samples were further purified using silver nitrate impregnated silica gel following the method proposed by D'Andrea et al. [9] and modified by Richter et al. [4]. The ketone fraction was cleaned up with DCM (100 %) followed by ethyl acetate (100 %), with the alkenones eluting in the last fraction.

2.2. Alkenone analysis

The alkenone fractions were analyzed using gas chromatography with a flame-ionization detector (GC-FID). Prior to the first analysis, 18-pentatriacontanone was added to the samples as an internal standard for quantification.

GC-FID analyses were performed on an Agilent 7890B using hydrogen as the carrier gas. The GC method used a splitless injection (320 °C). Two methods were used with two different GC columns as summarized in Table 1: VF1 method used the Agilent VF-200 ms column (60 m × 250 µm×0.10 µm), while the Rtx method used the Restek Rtx-200 column (105 m × 250 µm×0.25 µm) as described by Zheng et al. [13]. There are no significant differences between the two methods for our samples; thus the results from both GC methods can be compared without bias (Section A.1, Table A.1). The 9 samples were analyzed before and after saponification with the VF1 method. 5 samples were analyzed before and after purification with the Rtx method and the remaining 4 samples with the VF1 method.

A culture of Group II *Ruttnera lamellosa* RCC3687 was used as a standard and injected every 8 to 10 samples to assess the analytical precision. The standard deviation for the calculated U^K_{37} (see Eq. (2)) was 0.0041 (n = 7) for the VF1 method and 0.013 (n = 11) for the Rtx method.

Alkenone identification was performed by comparing GC retention time with the above-mentioned alkenone standard and published data. To confirm alkenone identification, some samples were analyzed by gas chromatography–mass spectrometry (GC–MS). Samples were run on an Agilent 7890B gas chromatograph equipped with a VF-200 ms column (60 m × 250 µm×0.10 µm) coupled with an Agilent 5977B mass spectrometer. The oven program started at 40 °C, temperature increased to 255 °C at 20 °C/min, then to 315 °C at 2 °C/min and was hold isothermally for 10 min. The MS ionization energy was set to 70 eV with a scan range of 50–600 m/z.

In this study, we focus on C_{37} alkenones as C_{38} alkenones are not always fully separated and are often present in low concentrations in our samples.

2.3. Alkenone indices

The relative abundance of each C_{37} alkenone to the total abundance of C_{37} alkenones was calculated as proposed by Rosell-Melé [14]:

$$\%C_{37:m} = 100 \times ([C_{37:m}] / [\text{sum of } C_{37} \text{ alkenones}]), \quad (1)$$

where m refers to the number of double bonds, which range from 2 to 4.

The alkenone unsaturation indices U^K_{37} [1] and $U^{K'}_{37}$ [15] were adapted for lacustrine environments by including both $C_{37:3a}$ and $C_{37:3b}$ isomers [e.g., 6] and calculated together with the $U^{K''}_{37}$ [16]:

$$U^K_{37} = \frac{[C_{37:2}] - [C_{37:4}]}{[C_{37:2}] + [C_{37:3a}] + [C_{37:3b}] + [C_{37:4}]} \quad (2)$$

$$U^{K'}_{37} = [C_{37:2}] / ([C_{37:2}] + [C_{37:3a}] + [C_{37:3b}]) \quad (3)$$

$$U^{K''}_{37} = [C_{37:4}] / ([C_{37:3a}] + [C_{37:3b}] + [C_{37:4}]) \quad (4)$$

Table 1
Parameters of the two GC-FID methods used for alkenone analysis.

Method	Column	Flow rate (ml/min)	Injected volume (µl)	Oven program
VF1 (Longo et al.,2013)	Agilent VF-200ms (60 m × 250 µm × 0.10 µm)	3.0	5	Initial temp 50 °C Ramp 20 °C/min to 235 °C Ramp 1.39 °C/min to 320 °C (held 5 min)
Rtx (Zheng et al.,2017)	Restek Rtx-200 (105 m × 250 µm × 0.25 µm)	1.5	1	Initial temp 50 °C (held 2 min) Ramp 20 °C/min to 255 °C Ramp 3 °C/min to 320 °C (held 25 min)

Table 2
Calculations used to assess the impact of saponification and silver-nitrate purification on the C_{37} alkenone relative abundances, the C_{37} alkenone-based indices, the temperature reconstructed from the U^K_{37} index using the calibration from D'Andrea et al. [18], the C_{37} alkenone peak area and the C37 alkenone concentrations.

	C_{37} alkenone-basedindices	Peak area change	C_{37} concentration
Saponification	$\Delta X = \lvert X_{\text{before saponification}} - X_{\text{after saponification}} \rvert$	$\Delta A(C_{37:m}) = [A(C_{37:m})_{\text{after saponification}} / A(C_{37:m})_{\text{before saponification}}] \times 100$	$\Delta(C_{37}) = [(C_{37})_{\text{after saponification}} / (C_{37})_{\text{before saponification}}] \times 100$
Purification	$\Delta X = \lvert X_{\text{before purification}} - X_{\text{after purification}} \rvert$	$\Delta A(C_{37:m}) = [A(C_{37:m})_{\text{after purification}} / A(C_{37:m})_{\text{before purification}}] \times 100$	–
Control	$\Delta X = \lvert Xt_1 - Xt_2 \rvert$	$\Delta A(C_{37:m}) = [A(C_{37:m})t_2 / A(C_{37:m})t_1] \times 100$	$\Delta(C_{37}) = [(C_{37})t_2 / (C_{37})t_1] \times 100$

$X = \%C_{37:m}$ with m varying from 2 to 4, or U^K_{37}, $U^{K'}_{37}$, $U^{K''}_{37}$, RIK_{37} or T.

Finally, the isomeric ratio of ketones [17] was calculated:

$$RIK_{37} = [C_{37:3a}]/([C_{37:3a}] + [C_{37:3b}]) \quad (5)$$

2.4. Comparison methodology

For each sample, the C_{37} alkenone relative abundances, indices and concentrations ($C_{37} = C_{37:2} + C_{37:3a} + C_{37:3b} + C_{37:4}$) were calculated before and after each clean-up step (Table 2). The absolute values of the differences between the alkenone abundances and indices before and after each clean-up step were calculated; a ratio was also calculated between alkenone concentrations before and after saponification (the standard was lost during the purification as it eluted in another fraction than the one with the alkenones). For each chemical step, all the differences and ratios obtained for the 9 samples were compared with the differences and ratios obtained for the control. For the control, which aims to assess the repeatability of the measurements, we analyzed the 9 samples twice, a few days apart, at the same chemical step. Then, we calculated the differences between the alkenone abundances and indices of the two analyses and the ratios of concentrations as described above (Table 2).

To understand how the two chemical steps influenced each of the C_{37} alkenones, for each sample, we compared the area of the peaks of each C_{37} alkenone before and after each clean-up step (Table 2). The change of the area (ΔA) calculated as the ratio of the area after the chemical step to the area before the chemical step indicates the proportion of each C_{37} alkenone recovered after the chemical step and the proportion lost (1 - ΔA). The same calculations were made for the control to assess the instrument uncertainty (Table 2).

In order to assess the impact that the clean-up steps could have on reconstructed temperatures, the U^{K}_{37} values of each sample were converted into temperatures using the calibration from D'Andrea et al. [18] obtained from lake Vikvatnet in Norway as an example.

2.5. Statistical analysis

Statistical analyses were performed using the nonparametric test of Mann-Whitney with Past statistical program v4.1 [19]. A p value of less than 0.05 was considered statistically significant.

3. Results and discussion

3.1. Impact of clean-up steps on alkenone distributions

3.1.1. Changes of C_{37} alkenone relative abundances

The means of the differences of C_{37} alkenone relative abundances before and after the saponification range from 1.1 ± 0.9 % for $\Delta\%C_{37:3a}$ to 3.5 ± 2.6 % for $\Delta\%C_{37:4}$ (Fig. 1, Table 3). These differences in alkenone relative abundances are higher than the ones of the control except for $\Delta\%C_{37:3a}$ (Fig. 1, Table 3). In particular, the differences of the relative abundance of $C_{37:4}$ alkenone before and after saponification is significantly higher than the ones of the control. Very similar results were obtained using methods with a reduced injection volume, showing that the high injection volume of the VF1 method (5 µl) did not significantly alter the results (Section A.2, Table A.2).

For purification, the means of the differences range from 1.7 ± 1.3 % for $\Delta\%C_{37:2}$ to 4.6 ± 2.6 % for $\Delta\%C_{37:4}$ (Fig. 1, Table 3). These differences are higher than the ones of the control, except for $\Delta\%C_{37:3b}$, and they are significantly higher than the ones of the control for the $C_{37:4}$ alkenone (Fig. 1, Table 3).

Both clean-up steps, saponification and silver-nitrate purification, significantly changed the C_{37} alkenone distribution. For both reactions, it is the $C_{37:4}$ alkenone which underwent the largest changes, followed by the $C_{37:3a}$, $C_{37:3b}$ and $C_{37:2}$ alkenones for purification, and the $C_{37:3b}$, $C_{37:2}$ and $C_{37:3a}$ for saponification.

3.1.2. Removing co-eluting peaks

The clean-up steps are used to remove co-eluting compounds which interfere with alkenone peaks. By doing so, they change the peak area of alkenones and thus their distribution. Indeed, saponification removed such co-eluting peaks for each of the 9 samples analyzed before and after saponification (Table 4). In all the samples, except for sample 4, saponification removed a peak eluting after the peak of the $C_{37:2}$ alkenone and co-eluting with it, as shown in Fig. 2a, b (Table 4, Appendix B). For sample 4, saponification removed a peak eluting between the peaks of the $C_{37:3b}$ and $C_{37:2}$ alkenones, and co-eluting with both alkenone peaks (Table 4, Appendix B). For samples 5, 8 and 9, a peak co-eluting with the peak of the $C_{37:3b}$ alkenone appeared after saponification (Table 4, Appendix B). For sample 3, the separation between the peaks of the $C_{37:3a}$ and $C_{37:3b}$ alkenones was improved after saponification (Table 4, Appendix B). For almost all of our samples, saponification was sufficient for cleaning C_{37} alkenone peaks for proper integration.

For each sample, we compared the area of each C_{37} alkenone peak before and after saponification (ΔA, see Table 2). The alkenone peaks which underwent a significant change due to saponification (as described above, a co-eluting peak was removed or appeared, or the separation from another alkenone peak was notably improved) are those showing the biggest or the smallest change (Fig. 3A, Table 4), depending on whether the change led to an increase or a decrease of the alkenone peak area. For each sample, the other C_{37} alkenones, those which did not

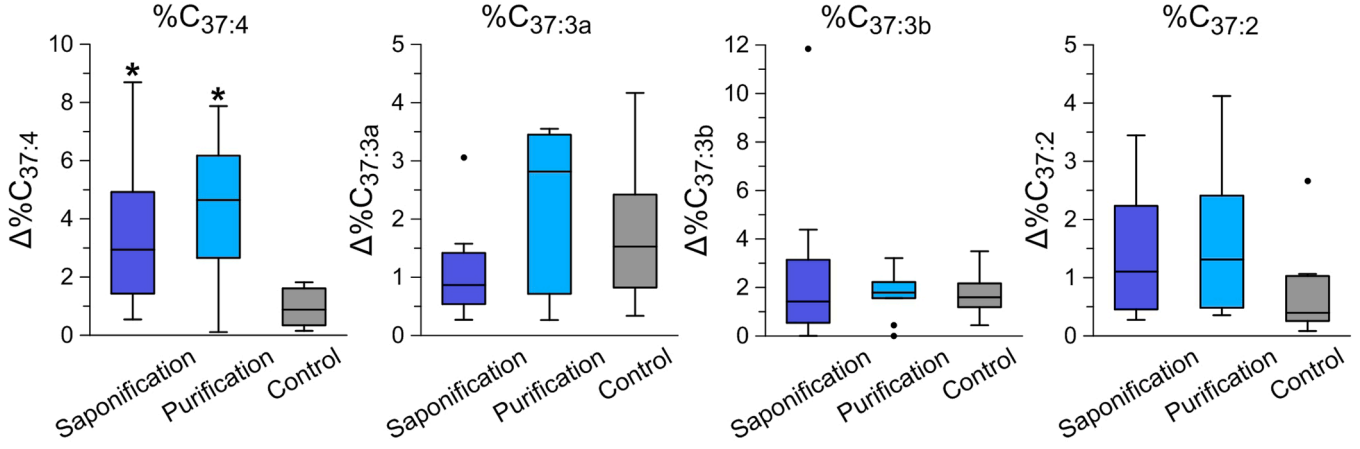

Fig. 1. Impact of clean-up steps on the C_{37} alkenone distributions. Differences in C_{37} alkenone relative abundances between samples analyzed before and after saponification ($n = 9$, dark blue) and before and after purification ($n = 9$, light blue), and samples analyzed twice, a few days apart, at the same chemical step, which constitute the control ($n = 9$, gray). The bottom and top lines of the box plot indicate the upper and lower quartiles of a sample set and solid lines inside the box, the median value. Outliers are shown by black dots. Significant difference with the control based on Mann-Whitney test is represented by * for p-value < 0.05.

Table 3
Differences in C_{37} alkenone relative abundances and C_{37} alkenone-based indices, and C_{37} alkenone concentration changes obtained comparing samples analyzed before and after saponification, before and after purification, and control samples analyzed twice, a few days apart, at the same chemical step (see Table 2). The means of the differences and ratios are provided with their standard deviations and the number of samples analyzed in each case. Mann-Whitney tests were performed to test if the differences for each calculated variable before and after each chemical process were significantly different from the control.

	$\Delta\%C_{37:4}$	$\Delta\%C_{37:3a}$	$\Delta\%C_{37:3b}$	$\Delta\%C_{37:2}$	ΔRIK_{37}	ΔU^{K}_{37}	$\Delta U^{K'}_{37}$	$\Delta U^{K''}_{37}$	ΔC_{37}
Saponification ($n = 9$)									
Mean	3.5	1.1	2.7	1.4	0.03	0.04	0.02	0.04	54.2
SD	2.6	0.9	3.7	1.1	0.05	0.02	0.02	0.03	11.1
p Mann-Whitney	0.01*	0.3	0.7	0.2	0.3	0.002**	0.2	0.04*	0.0004***
Purification ($n = 9$)									
Mean	4.6	2.1	1.8	1.7	0.02	0.06	0.02	0.04	
SD	2.6	1.4	1.0	1.3	0.02	0.03	0.02	0.03	
p Mann-Whitney	0.006*	0.6	0.9	0.06	0.3	0.006*	0.6	0.01*	
Control ($n = 9$)									
Mean	0.9	1.8	1.8	0.8	0.03	0.01	0.01	0.01	99.6
SD	0.7	1.3	1.0	0.8	0.02	0.01	0.01	0.01	5.8

* *p*-value < 0.05.
** *p*-value < 0.005.
*** *p*-value < 0.0005.

Table 4
Changes of the peak area of each C_{37} alkenone resulting from saponification and silver-nitrate purification for each analyzed sample (see Table 2). The standard deviation of the peak area changes is calculated for each sample considering all C_{37} alkenone (SD) and removing the changes associated with the removal or appearance of a co-eluting peak, or notable improvement of the separation between two peaks (**SD**). The peak area changes normalized by the peak area change of the $C_{37:2}$ alkenone are given for purification. The GC method used is mentioned for each sample.

Sample	Method	$\Delta A(C_{37:4})$	$\Delta A(C_{37:3a})$	$\Delta A(C_{37:3b})$	$\Delta A(C_{37:2})$	SD	SD[d]	$\Delta A(C_{37:4})/\Delta A(C_{37:2})$	$\Delta A(C_{37:3a})/\Delta A(C_{37:2})$	$\Delta A(C_{37:3b})/\Delta A(C_{37:2})$
Saponification ($n = 9$)										
1	VF1	47	43	41	**34**[a]	5.4	3.3			
2	VF1	33	30	28	**35**[a]	3.2	2.7			
3	VF1	45	41	**32**[c]	**39**[a]	5.6	3.1			
4	VF1	63	56	**47**[b]	**35**[a]	12.1	4.8			
5	VF1	54	52	51	**46**[a]	3.3	1.6			
6	VF1	83	74	79	**89**[a]	6.1	4.6			
7	VF1	51	52	55	**58**[a]	3.2	2.1			
8	VF1	92	83	**84**[b]	**82**[a]	4.5	4.6			
9	VF1	41	33	**18**[b]	**41**[a]	10.9	5.3			
Mean		*57*	*52*	*48*	*51*	*6.0*	*3.6*			
Purification ($n = 9$)										
1	Rtx	37	47	49	45	5.3	5.3	82	104	109
2	Rtx	34	40	43	49	6.1	6.1	70	81	88
3	Rtx	39	53	52	69	12.2	12.2	56	77	75
4	Rtx	40	46	50	87	21.4	21.4	46	53	57
5	Rtx	34	45	48	52	5.1	5.1	66	87	92
6	VF1	63	58	**64**[a]	71	7.6	6.3	89	82	90
Mean ($n = 6$)		*41*	*48*	*51*	*62*	*9.6*	*9.4*	*68*	*81*	*85*
7	VF1	73	74	71	71	1.6	1.6	103	105	101
8	VF1	72	61	75	**53**[a]	10,0	7.5	135	114	141
9	VF1	69	59	**73**[c]	62	6.1	4.7	110	95	117
Mean ($n = 9$)		*51*	*54*	*58*	*62*	*8.4*	*7.8*	*84*	*89*	*97*
Control ($n = 9$)										
Mean		*86*	*90*	*87*	*88*	*8.8*				
SD		*18*	*20*	*26*	*19*					

Bold numbers are used when the clean-up step significantly changed an alkenone peak by:
[a] Standard deviation of $\Delta A(C_{37:m})$ for each sample when the alkenones concerned by a significant change due to the clean-up step were excluded.
[b] removal of a compound co-eluting with the concerned alkenone,
[c] appearance of a compound co-eluting with the concerned alkenone,
[d] notable improvement of the separation between two peaks.

experience a significant change due to saponification, show similar changes of their peak area. This is reflected by the reduction of the standard deviations when peaks which underwent a significant change due to saponification were excluded (mean of standard deviations of 3.6, Table 4). Even if these peak area changes are similar, the $C_{37:4}$ alkenone was the most conserved alkenone on average (Fig. 3A, Table 4). The $C_{37:4}$ alkenone is also the most abundant alkenone; therefore, when considering the relative abundances of C_{37} alkenones, it is the one showing the largest changes among the C_{37} alkenones while it is the one that changed the least with saponification (Table 3).

These observations show that saponification had an equal effect on every C_{37} alkenone expect when saponification led to a significant change in alkenone peak area due to the removal of a co-eluting peak in most cases. Therefore, the distribution changes resulting from

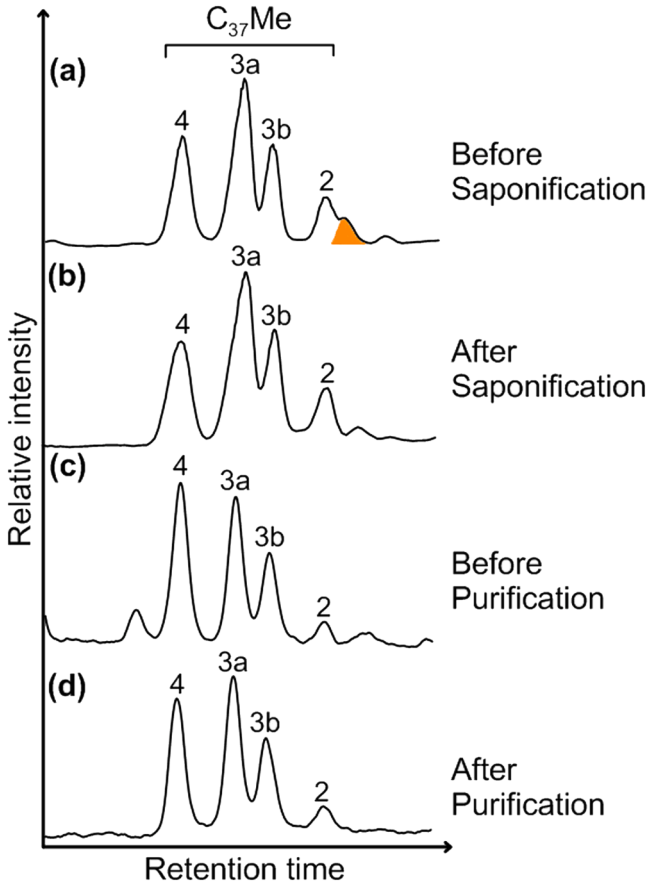

Fig. 2. Comparison of GC-FID chromatograms at various chemical steps. Sample 7 (a) before saponification: a peak co-elutes with the $C_{37:2}$ alkenone (colored in orange); (b) after saponification: the co-eluting peak has been removed. Sample 3 (c) before silver-nitrate purification: the peak of $C_{37:4}$ alkenone is higher than the one of $C_{37:3a}$ alkenone, no visible co-eluting peak; (d) after purification: the peak of $C_{37:3a}$ alkenone is higher than the one of $C_{37:4}$ alkenone.

saponification seem to be mainly caused by the removal of co-eluting peaks, that is to say, the removal of biases in the signal. In most cases, saponification improved the signal without significantly altering the original C_{37} alkenone distribution.

3.1.3. *A differential impact based on unsaturation number?*

On the other hand, the purification process using silver nitrate removed visible co-eluting peaks in the elution zone of C_{37} alkenone peaks in only 2 out the 9 samples analyzed before and after purification (Table 4, Appendix C). This results from the fact that for the studied samples, saponification was often enough to remove all the co-eluting peaks in the C_{37} alkenone elution zone. However, silver-nitrate purification was often efficient in removing co-eluting peaks in the elution zone of the C_{38} alkenones (Appendix C). For sample 9, silver-nitrate purification seems to have improved the separation between the peak of the $C_{37:3b}$ alkenone and a co-eluting peak (Appendix C).

While for the majority of the samples, there was no visible co-eluting peak interfering with the C_{37} alkenones to remove, silver-nitrate purification significantly modified C_{37} alkenone distributions. Notably, for some samples, the purification led to an inversion of the order of abundance of the $C_{37:4}$ and $C_{37:3a}$ alkenones: before the purification the $C_{37:4}$ was the most abundant while after the purification, the $C_{37:3a}$ became the most abundant, as shown in Fig. 2c, d.

The changes in the area of each C_{37} alkenone peak for each sample before and after purification (ΔA, see Table 2) were more heterogeneous between alkenones compared to saponification, as shown by the higher values of the standard deviation even when the peaks which underwent the removal a co-eluting peak or a notable improvement of the separation from a co-eluting peak were excluded (mean of the standard deviations of 8.4 and 7.8, respectively, versus 6.0 and 3.6 for saponification, Table 4). Sample 7 appears to be an exception, where the loss in alkenone peak area appears to be homogeneous across the C_{37} alkenones, with ~30 % loss for each peak (Fig. 3B, Table 4).

Calculating the average of the area changes for each C_{37} alkenone peak for the 9 purified samples, it appears that the most important reduction of the area occurred for the $C_{37:4}$ alkenone (49 %), then for the $C_{37:3a}$ (46 %), the $C_{37:3b}$ (42 %) and finally, the $C_{37:2}$ alkenone (38 %, Table 4). Normalizing the C_{37} alkenone peak area changes by the peak area change of the most conserved alkenone, the $C_{37:2}$ alkenone, such general pattern of increased loss of peak area with the unsaturation number of alkenones is found for the samples 1 to 6 (Fig. 3B, Table 4).

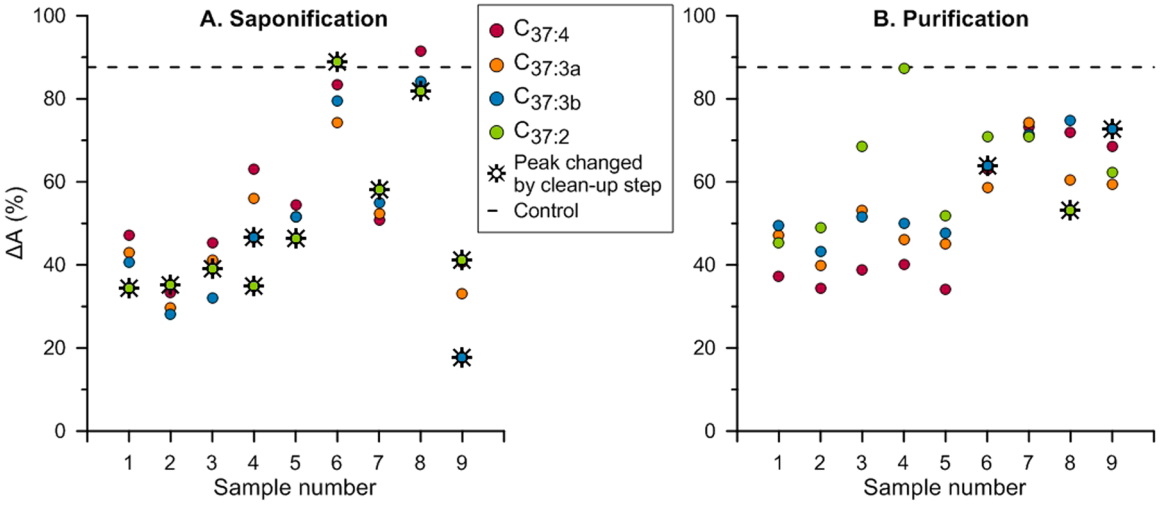

Fig. 3. Impact of clean-up steps on C_{37} alkenone peak area. Changes of the peak area of each C_{37} alkenone, $C_{37:4}$ (pink dots), $C_{37:3a}$ (orange dots), $C_{37:3b}$ (blue dots) and $C_{37:2}$ alkenones (green dots), resulting from saponification (A) and silver-nitrate purification (B) for each analyzed sample (see Table 2). 100 % indicates that the total alkenone peak is recovered after the clean-up step. The average area change obtained for all C_{37} alkenone peaks for the control is displayed (black dashed line). When the clean-up step significantly changed an alkenone peak by the removal or appearance of a co-eluting compound or the notable improvement of the separation between two peaks, the dot of the concerned alkenone is surrounded by a crown.

For these samples, the purification led to a higher loss of the $C_{37:4}$ than the $C_{37:2}$ alkenone (on average, 32 % of additional loss for the $C_{37:4}$ compared to the $C_{37:2}$ alkenone, Table 4). The $C_{37:3a}$ and $C_{37:3b}$ alkenones of these 6 samples had similar losses, which are greater than the loss of the $C_{37:2}$ alkenone, except for sample 1 (on average, 19 % and 15 % of additional loss, respectively, Table 4).

The silver-nitrate purification was first used by D'Andrea et al. [9] to separate alkenones based on their number of double bonds. The silver nitrate has an affinity for electrons on the double bonds resulting in a higher retention power of unsaturated compounds [9]. The silver-nitrate purification was then modified and used as a process to remove co-eluting peaks resistant to saponification [e.g., 4]. However, no extensive study demonstrated that this clean-up step does not have an impact on the alkenone distribution for lacustrine samples. Our observations show that the silver-nitrate purification altered the C_{37} alkenone distribution by increasing the loss of alkenones with a higher number of double bonds.

However, this differential impact of silver-nitrate purification depending on the number of double bonds was not observed for all samples. The losses of samples 7–9 do not show any pattern linked with the number of double bonds (Fig. 3B, Table 4). For sample 8, the fact that purification removed a peak co-eluting with the $C_{37:2}$ alkenone may have altered the possible link between the losses and the number of double bonds.

This different response to the purification process does not seem to be linked with the concentration of alkenones before purification (Fig. A.1); this discards the hypothesis of silver nitrate impregnated silica gel column overloading. It seems unlikely that the difference could arise from the different methods used as sample 6, which was analyzed with the VF1 method, experienced greater losses for the $C_{37:4}$, $C_{37:3a}$ and $C_{37:3b}$ alkenones than for the $C_{37:2}$ alkenone as the samples analyzed with the Rtx method (Table 4). Moreover, VF1 and Rtx methods were shown to provide equivalent results (Section A.1, Table A.1). Samples 7 and 9 appear to contain a mixture of Group I/Group II alkenones and sample 8 has a RIK_{37} value at the limit of the Group I range, but this should not have any impact. The difference of behavior towards purification could result from lake-specific effects linked with the different matrices.

Even if silver-nitrate purification can be efficient in removing co-eluting peaks in the C_{37} and C_{38} alkenone elution zone, this process should be used with caution, and paying particular attention to changes in alkenone distributions before and after purification.

A new method using silver thiolate flash column chromatography was proposed by Wang et al. [7] to replace the purification process using silica gel impregnated with silver nitrate. This new method allows for a more efficient separation of alkenones from co-eluting compounds [7] but there is no extensive investigation of the impact on alkenone distribution and C_{37} alkenone-based indices of lacustrine sediments.

3.2. Impact on C_{37} alkenone-based indices

The means of the differences of the U^K indices before and after saponification and before and after purification are higher than the ones of the control (Fig. 4, Table 3). For saponification, they range from 0.02 ± 0.02 for $\Delta U^{K'}_{37}$ to 0.04 ± 0.02 for ΔU^{K}_{37} and 0.04 ± 0.03 for $\Delta U^{K''}_{37}$, and for purification, from 0.02 ± 0.02 for $\Delta U^{K'}_{37}$ to 0.06 ± 0.03 for ΔU^{K}_{37} (Fig. 4, Table 3). The differences are significantly higher than the ones of the control for U^{K}_{37} and $U^{K''}_{37}$ for both purification and saponification (Fig. 4, Table 3). The differences for the RIK_{37} index are equal to the control for saponification and slightly lower than the control for purification (Fig. 4, Table 3).

As the relative abundance of the $C_{37:4}$ alkenone was the most impacted by saponification and purification (see Sections 3.1.2. and 3.1.3.), the U^{K}_{37} and $U^{K''}_{37}$indices were affected since this alkenone is included in the equations (Eqs. (2) and (4)). The $U^{K'}_{37}$ and RIK_{37} indices do not contain the $C_{37:4}$ alkenone in their equations (Eqs. (3) and (5)) and thus they underwent less change. Villanueva et al. [20] and Gal et al. [21] also found that saponification did not significantly alter the $U^{K'}_{37}$ index and the estimated sea surface temperatures for marine sediments.

The means of the differences of the temperatures reconstructed based on the calibration from D'Andrea et al. [18] are 1.5 ± 0.9 °C for saponification and 2.2 ± 1.2 °C for silver-nitrate purification. These differences are significantly higher ($p = 0.002$ for saponification and $p = 0.006$ for purification) relative to the ones of the control (0.4 ± 0.3 °C). As seen in Section 3.1.2, the alkenone distribution changes occurring during saponification mainly resulted from the removal of co-eluting compounds interfering with alkenones thus, saponification would have avoided a bias of 1.5 °C on average. On the other hand, silver-nitrate purification altered the alkenone distribution of 6 samples (see Section 3.1.3), which would have resulted in a bias of 2.9 °C on average. The changes experienced by the 3 other samples during purification, resulting only partly from the removal of a co-eluting peak or the notable improvement of the separation from a co-eluting peak, would have been associated with a change of reconstructed temperature of 0.8 °C on average.

When possible, it would be better to reduce chemical preparation to limit the risk of modifying alkenone distributions. Indeed, an inter-laboratory comparison study on the $U^{K'}_{37}$ index carried out by Rosell-Melé et al. [22] noticed that adding clean-up steps can lead to a larger

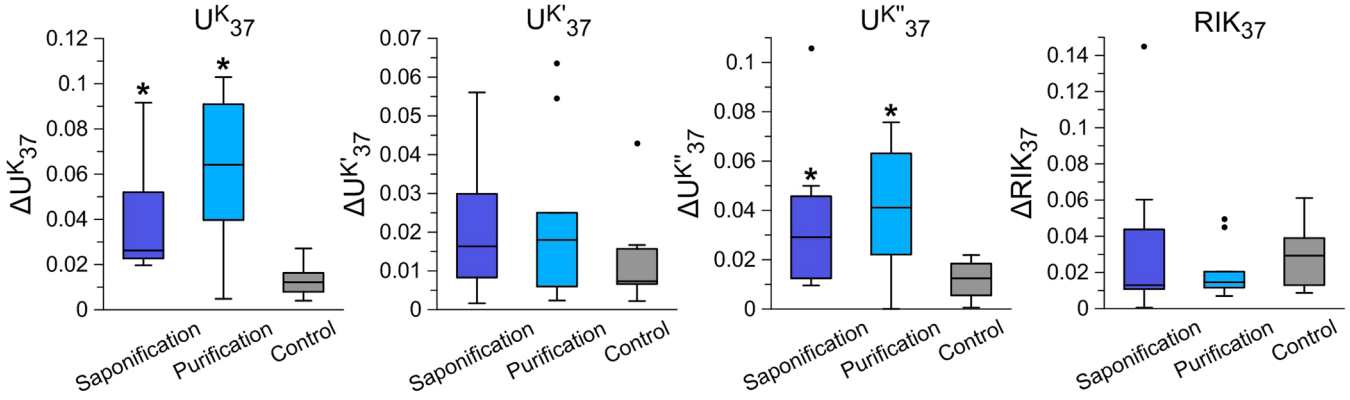

Fig. 4. Impact of clean-up steps on C_{37} alkenone-based indices. Differences in C_{37} alkenone-based indices between samples analyzed before and after saponification ($n = 9$, dark blue) and before and after purification ($n = 9$, light blue), and samples analyzed twice, a few days apart, at the same chemical step, which constitute the control ($n = 9$, gray). The bottom and top lines of the box plot indicate the upper and lower quartiles of a sample set and solid lines inside the box, the median value. Outliers are shown by black dots. Significant difference with the control based on Mann-Whitney test is represented by * for p-value < 0.05.

scattering of the results. If a clean-up step is needed, saponification was sufficient to remove compounds co-eluting with C_{37} alkenones in most of our samples. Until silver-nitrate purification is improved and specific tests are conducted on diverse lacustrine samples, this process should be used only if necessary and checking that it does not alter the alkenone distribution. Another possibility would be to use novel methods such as reversed-phase high pressure liquid chromatography-mass spectrometry [23,24] which provides an efficient separation of alkenones from the co-eluting compounds without additional clean-up steps.

3.3. Effects on alkenone concentration

The mean of the ratios of C_{37} alkenone concentrations obtained before and after saponification is 54 ± 11 %: after saponification, the C_{37} alkenone concentrations were, on average, reduced almost by half compared to the ones obtained before saponification (Table 3). This result is significantly smaller than the control (99.6 ± 6 %, Table 3). We found a very similar result of 52 ± 20 % of recovery when averaging the area changes due to saponification of all the C_{37} alkenones for the 9 studied samples. The silver-nitrate purification resulted in a similar 56 ± 14 % of C_{37} alkenone recovery on average, considering all the samples. These figures are higher than the control whose average area change is 88 ± 20 %.

Each clean-up step resulted in a loss of almost half of the C_{37} alkenone concentration. Gal et al. [21] already noted a concentration loss of about 20 % due to saponification in marine sediments. As Villanueva et al. [20] showed no impact of saponification on alkenone concentrations in marine sediments, Gal et al. [21] suggested that this loss was linked with the elimination of interfering compounds by saponification. However, Rosell-Melé et al. [22] found large differences in the alkenone concentrations in their interlaboratory comparison, which could be linked to the use of clean-up steps – including saponification – by some laboratories. In our case, it seems very unlikely that the loss of almost half of the concentration is attributable to the removal of co-eluting compounds. Part of the loss attributed to saponification could also come from the process of separation of the different fractions, which followed the saponification. Lacustrine sediments could also be more affected by saponification than marine sediments, possibly due to the different matrix effects.

The recovery percentages of alkenones are quite variable depending on samples. This variability is weakly linked with the alkenone concentration before saponification (R^2 = 0.12, Fig. A.2) and the silver-nitrate purification (R^2 = 0.27 for all the C_{37} alkenones, and ranging from 0.07 to 0.40 for individual C_{37} alkenones, all excluding sample 6, see Fig. A.3) with the loss increasing with alkenone concentration present before the clean-up step. Lake-specific effects may be responsible for such variability.

4. Conclusions

We noticed that C_{37} alkenone abundances, indices and concentrations were not very sensitive to the use of the Agilent VF-200 ms (60 m × 250 µm×0.10 µm) or the Restek Rtx-200 (105 m × 250 µm×0.25 µm) columns or to injection volume changes.

On the other hand, the clean-up steps used for sample preparation significantly changed the C_{37} alkenone distribution and quantification as well as the C_{37} alkenone-based indices. Saponification modified the C_{37} alkenone distribution but these changes seemed to mainly result from the removal of co-eluting compounds, improving the quality of the results and avoiding a bias corresponding to 1.5 °C on average. The purification process using silver nitrate impregnated silica gel altered the C_{37} alkenone distribution of 6 out of the 9 studied samples by preferentially retaining alkenones with more double bonds. This resulted in changes of the C_{37} alkenone-based indices, in particular the U^{K}_{37} and $U^{K'}_{37}$ indices, which would be associated with an average error of 3 °C in reconstructed temperatures.

Both saponification and silver-nitrate purification had a strong impact on C_{37} alkenone concentration, which were, on average, almost reduced by half after each clean-up step.

Clean-up steps for sample preparation should be used with caution, in particular silver-nitrate purification, which should be preferably avoided when possible (saponification was enough to clean the C_{37} alkenones for most of the studied samples) and only applied when changes in alkenone distributions are carefully monitored. It could be advantageous to test novel methods using reversed-phase high pressure liquid chromatography, which allow for better separation efficiency without any supplementary chemical processes.

This study highlights that alkenone analysis in lake sediments is still not a routine process. This originates from the fact that lacustrine matrices can be complex and variable from one lake to another, and also because the analytical methods recently developed are generally tested only on a few lake samples. Therefore, we recommend caution when analyzing alkenones from lacustrine samples and to rigorously test existing clean-up methods before applying them to new lakes and paleoreconstructions.

CRediT authorship contribution statement

Céline Martin: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Nora Richter:** Methodology, Validation, Writing – review & editing. **Ronald Lloren:** Data curation, Formal analysis, Writing – review & editing. **Nathalie Dubois:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Most of the data are available in the manuscript, the rest will be available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2023.464576.

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