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Organic matter removal for continuous flow isotope ratio mass spectrometry analysis of carbon and oxygen isotope compositions of calcite or dolomite in organic-rich samples

Carine Chaduteau,[1] Magali Ader,[1]* Oanez Lebeau,[2] Guillaume Landais,[1] Vincent Busigny[1,3]

[1]Université de Paris, Institut de Physique du Globe de Paris, CNRS, Paris, France
[2]IUEM, Laboratoire Domaines Océaniques, Université de Bretagne Occidentale, Plouzane, France
[3]Institut Universitaire de France, Paris, France

Abstract

$\delta^{13}C$ and $\delta^{18}O$ values of carbonate in organic-rich samples such as soils, biofilms, and lake sediments have been scarcely used so far in paleo-environmental, biomineralization, or diagenetic studies because organic matter in high proportions is suspected to alter carbonate isotope analysis. Yet, with the improvement of analytical capabilities and in particular the use of CF-IRMS, this may not be an issue anymore. To evaluate this, $\delta^{13}C$ and $\delta^{18}O$ values of calcite or dolomite mixed in various proportions with yeast (used here as a model for immature organic matter) were measured. The results indicate that measurements of calcite or dolomite $\delta^{13}C$ and $\delta^{18}O$ by CF-IRMS require organic matter removal only when its weight proportion exceeds that of carbonates. Yeast generated an unidentified molecule during phosphoric acid digestion at 25°C (none at 80°C), which shifted the carbonate $\delta^{18}O$ values for yeast proportions higher than 50%. It also generated CO_2, but with a noticeable shift in $\delta^{13}C$ and $\delta^{18}O$ values only at 80°C (none at 25°C) and for yeast proportions higher than 75%. Three methods of organic matter removal were tested: the well-established NaOCl and H_2O_2 wet treatments as well as low temperature plasma ashing (LTA) so far seldom examined. LTA removed the isotope shifts, although imperfectly for yeast proportions of 95%, without carbonate dissolution. NaOCl efficiently removed the isotope shifts in all cases but dissolved part of the calcite and dolomite. H_2O_2 treatment caused severe dissolution of calcite and dolomite while worsening the $\delta^{13}C$ and $\delta^{18}O$ shifts and should thus be avoided.

Carbon and oxygen isotope analyses of calcite ($CaCO_3$) and dolomite ($CaMg[CO_3]_2$) in sediments, soils, shells, speleothems, microbialites, and sedimentary rocks are routinely performed to reconstruct paleo-environmental changes or diagenetic history. In most cases, these analyses do not require pretreatment to remove organic matter because carbonates are in large excess relative to organic matter (e.g., Wierzbowski 2007). Pretreatments remain however sometimes necessary, especially when samples contain immature organic matter. CO_2 and/or other molecular gaseous species may be produced during the reaction of organic matter with orthophosphoric acid (H_3PO_4) (Bowen 1966, 1991; Epstein et al. 1951, 1953; Falster et al. 2018; Oehlerich et al. 2013; Weber et al. 1976) and their incomplete separation prior to gas introduction in the mass spectrometer may affect C and O isotope measurements. Various treatments for organic matter removal have thus been tested (*see* reviews in Grottoli et al. 2005; Keatings et al. 2006; De Groot 2011; Falster et al. 2018), for instance in the view of defining standard procedures for C and O isotope analyses of foraminifera (e.g., Feldmeijer et al. 2013), ostracod (e.g., Keatings et al. 2006), corals, and/or coccolithophores (e.g., Stevenson et al. 2014). However, these treatments were tested only for relatively low organic matter to carbonate ratios (<15%, Wierzbowski 2007), typical of most geological samples, except for H_2O_2 treatment, tested recently by Falster et al. (2018) for extremely high ratios. Although they are not very common in sedimentary rocks, high ratios can be found in soils, in biofilms or more generally in organic-rich and/or carbonate-poor marine or lacustrine sediments. In addition, the effect of organic matter removal treatments on carbonate isotope compositions has mostly been tested using vacuum CO_2 extraction and cryogenic purification systems such as vacuum lines (e.g., Wierzbowski 2007) or Kiel

*Correspondence: ader@ipgp.fr

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devices (e.g., Nagtegaal et al. 2012). Many laboratories now use continuous flow methods with a gas chromatographic column separation (CF-IRMS), which purifies CO_2 from other gases by gas chromatography rather than by cryogenic separation and may incompletely separate CO_2 from other contaminant gaseous species possibly generated during phosphoric acid attack. Last, all the recent CF-IRMS studies used a phosphoric acid digestion temperature ranging from 72°C to 90°C (Falster et al. 2018; Feldmeijer et al. 2013; Oehlerich et al. 2013; Keatings et al. 2006), i.e., quite high compared to the temperature of 25°C used for calcite in most earlier studies. Such high temperatures might increase the reactivity of organic matter with phosphoric acid.

In the present work, $\delta^{13}C$ and $\delta^{18}O$ values of calcite or dolomite mixed in various proportions with yeast (used here as a model for immature organic matter) were digested respectively at 25°C and 80°C and measured by CF-IRMS in order to evaluate the need for organic matter removal. The proportions of yeast included the extreme values of 75%, 90%, and 95% hardly ever investigated so far to our knowledge. Three widely used organic matter removal treatments were also tested and compared: wet oxidation with NaOCl (e.g., Charef and Sheppard 1984; Durazzi 1977; Emiliani 1966; Grottoli et al. 2005; Irwin et al. 1977; Land et al. 1975); wet oxidation with H_2O_2 (e.g., Boiseau and Juillet-Leclerc 1997; D'Eugenio and Leone 1989; D'Hondt and Lindinger 1994; McConnaughey 1989) also often used before Δ_{47} measurements (e.g., Grauel et al. 2013; Katz et al. 2017; Peral et al. 2018; Tripati et al. 2010) and dry oxidation using low-temperature oxygen-plasma ashing (LTA) (Conan and Brummer 2000; Erez and Honjo 1981; Goreau 1977; Hendry and Kalin 1997; Loncaric et al. 2007; Swart and Coleman 1980;). Roasting was not investigated here as several previous studies demonstrated that this treatment yields unsatisfactory results (e.g., Boiseau and Juillet-Leclerc 1997; Erez and Honjo 1981; Gaffey et al. 1991; Grottoli et al. 2005; Guiguer et al. 2003; Leone et al., 2000; Wierzbowski 2007). Phosphoric acid digestion was performed at 25°C for calcite samples and 80°C for dolomite samples, allowing to test the impact of temperature on the genesis of CO_2 and/or other contaminants from immature organic matter before and after treatment.

Materials and methods

Sample preparation

Two series of samples were prepared from variable proportions of either yeast and calcite or yeast and dolomite. The calcite (sample RENNES 0, labeled R_0) is a synthetic powder from Prolabo (99.5%), used in our laboratory as an internal isotope standard. The dolomite (sample INYO) is a natural sample of dolomitic marble from the Inyo mountains in California (USA). The dolomite was crushed to fine grain (<50 μm) using an agate mortar and thoroughly homogenized. Yeast was purchased in a 500 g box as dry balls of 1 mm diameter (cf. LEVURE-SECHE at "L'atelier de la patisserie"). It was also crushed to a fine powder in an agate mortar. Yeast (i.e., *Saccharomyces cerevisia*) was selected to represent immature organic matter because it contains all the molecular constituents of living organisms. It is assumed here to represent organic matter at the initial stages of early diagenesis. In addition, it can be easily purchased and preserved under dried and cooled conditions without any evolution of its C isotope composition. Variable amounts of carbonate and yeast were weighted and mixed directly in glass vials to obtain homogeneous synthetic samples with a yeast proportion of 10, 25, 50, 75, 90, and 95 wt%.

Treatments for organic matter removal

Wet oxidation by H_2O_2 or NaOCl

The wet oxidation reactions took place in a chemical fume hood at room temperature (21 ± 2°C). Aliquots of synthetic sample powders were placed into glass beakers with reagent grade hydrogen peroxide 30% (H_2O_2, pH = 3.8) (VWR Prolabo, AnalaR Normapur) or sodium hypochlorite (NaOCl, pH = 12) with 3.5% of active chlorine (VWR Prolabo, GPR Rectapur). Sample masses, reagent volumes, and reaction times are given in the Table S1. Two protocols were tested with H_2O_2 solution. The H_2O_2-A protocol corresponds to a maximum yeast proportion of 16.9 g per liter of H_2O_2 (i.e., 40 ml of H_2O_2 for 750 mg of yeast) and a reaction time of 24 h. The H_2O_2-B protocol corresponds to a maximum yeast proportion of 2.9 g per liter of H_2O_2 (i.e., 200 ml of H_2O_2 for 600 mg of yeast) and a reaction time of 72 h. Two protocols were tested for NaOCl solutions. The NaOCl-A protocol lasted 24 h with a maximum yeast proportion of 6.8 g per liter of NaOCl (i.e., 100 ml of NaOCl for 750 mg of yeast). The NaOCl-B protocol lasted 48 h with a maximum yeast proportion of 4.8 g per liter of NaOCl (i.e., 120 ml of NaOCl for 600 mg of yeast). After reaction, samples were centrifuged and rinsed five times with MilliQ water. Finally the residual powders were dried in an oven at 40°C for at least 2 d.

Dry oxidation by low temperature oxygen-plasma ashing

Aliquots of synthetic samples were spread in glass petri dishes and covered with riddled aluminum foil. Sample weight was adjusted so that it contained 15 mg of carbonates, a comfortable amount for subsequent isotopic analyses. The amount of yeast to be removed was thus comprised between 0 and 285 mg. The petri dishes were placed in a low-temperature oxygen-plasma asher (Polaron PT7160 RF) and evacuated until 10^{-1} mbar. Oxygen was then injected in the asher, and an electric discharge lighted the plasma. This plasma was made of free oxygen radicals, which oxidized organic matter to CO_2. The treatment was initially applied for 1–8 h depending on the amount of yeast and the powder thickness in the petri dishes. A few samples were treated again for up to seven additional hours.

Carbonate quantification and carbon and oxygen isotope analyses

Carbonate contents as well as carbon and oxygen isotope compositions of treated and untreated samples were measured by CF-IRMS. Both an Analytical Precision 2003 (AP2003) and

a Gas Bench coupled with a Delta plus XP were used in this study.

The weighed samples were placed in 12 ml borosilicate glass vials (Labco exetainer), sealed with a rubber septum and flushed with helium 5.6 (>99.9996%). About 0.1 ml of 100% H_3PO_4 was manually injected using a syringe. Phosphoric acid was prepared without potassium dichromate ($K_2Cr_2O_7$), to avoid any production of CO_2 by yeast oxidation with $K_2Cr_2O_7$ (Sarkar et al. 1990). The samples were reacted for 4 h at 25°C for calcite and 2 h at 80°C for dolomite.

For measurements using the AP2003, the gas was sampled automatically from vial headspace by a Gilson 22X autosampler equipped with a capillary needle. The gas was transferred by helium continuous flow and passed through a Nafion membrane to remove H_2O. It was then introduced in a chromatographic column (stainless column 0.3 m × 1/8″ × 2 mm, packed with Hayesep Q 60/80 mesh, Chrompack) in order to separate CO_2 from other gas components (e.g., N_2, O_2, Ar). The CO_2 was finally transferred to the mass spectrometer. The $\delta^{13}C_{CO2}$ and $\delta^{18}O_{CO2}$ values of the sample CO_2 were calculated relative to a CO_2 reference gas of 5.2 purity grade (>99.9992%) injected between each sample peak. For each vial, four successive analyses were carried out and averaged to provide the δ values.

The Gas Bench coupled with a Delta plus XP was operated according to the same principle as the AP2003 with only minor differences. The gas from the vials was sampled automatically via a CTC Combi-Pal autosampler. The gas chromatography column was a fused silica Poraplot Q column (25 m × 0.32 mm) heated to 70°C. Each IRMS measurement cycle started by five injections of CO_2 reference gas, followed by six aliquots of each sample.

For both mass spectrometers, measurements were performed in batches, which included several sets of internal standards. Standards of pure carbonate (RENNES 0 or INYO) of identical weight were placed every six samples to check the absence of drifts. A set of six standards of various weights of RENNES 0 or INYO was used to calibrate the CO_2 signal intensity relative to the carbonate amount, allowing the quantification of the carbonate content of each sample. The external precision (2σ) for the carbonate content estimated from replicate analyses of standards was ±10% relative to the measured value. A set of three calcite internal standards with various isotopic compositions was used to calibrate the $\delta^{13}C$ and $\delta^{18}O$ values relative to PDB (Pee Dee Belemnite). We report the $\delta^{13}C$ and $\delta^{18}O$ values of the CO_2 produced by phosphoric acid attack ($\delta^{18}O_{CO2}$ and $\delta^{13}C_{CO2}$) rather than those of carbonate. This allows us to perform mass and isotope balance calculation showing that part of the sample's CO_2 was derived from the partial oxidation of yeast (*see* first section of the discussion). The external precision (2σ) estimated from replicate analyses on RENNES 0 ($n = 32$) was ±0.14‰ and ±0.10‰ for $\delta^{13}C_{CO2}$ and $\delta^{18}O_{CO2}$ respectively. Each synthetic sample was measured at least four times (i.e., twice in two different batches).

The $\delta^{13}C_{CO2}$ and $\delta^{18}O_{CO2}$ values of calcite internal standards, RENNES 0 and INYO, were measured using the classical method of McCrea (1950) in order to calibrate them against the NBS-19 and IAEA-CO-1 international standards. The McCrea method was applied as follows. The sample was reacted with phosphoric acid under vacuum at 25°C for calcite for 4 h and at 80°C for 2 h for dolomite. The evolved CO_2 was then cryogenically purified in a vacuum line using a temperature controlled liquid-N_2 trap set at −130°C and then manometrically quantified. Its isotope composition was analyzed with Delta Plus XP or MAT253 mass spectrometers in Dual Inlet mode. The $\delta^{13}C_{CO2}$ and $\delta^{18}O_{CO2}$ values of a few untreated calcite-yeast mixture samples were also analyzed using this method in order to compare cryogenic purification on vacuum line with gas chromatography separation.

The $\delta^{13}C$ value of yeast was measured using sealed-tube combustion with copper oxide (i.e., Dumas combustion), offline vacuum cryogenic purification and quantification of CO_2, and isotope measurement on a Delta Plus XP mass spectrometer (for more details on the method, *see* Ader et al., 1998).

Results

The main goal of the present work is to assess the difference in $\delta^{13}C_{CO2}$ and $\delta^{18}O_{CO2}$ measured for synthetic mixtures of organic carbon and carbonate from values expected in pure carbonate standards (i.e., RENNES 0 or INYO). The results for calcite and dolomite are reported in Tables 1 and 2, respectively. The isotope deviations of the sample mixtures from the pure standard carbonates (also called isotope shifts) are expressed as $\Delta^{13}C$ and $\Delta^{18}O$, with $\Delta^{13}C = \delta^{13}C_{CO2\text{-sample}} - \delta^{13}C_{CO2\text{-standard}}$ and $\Delta^{18}O = \delta^{18}O_{CO2\text{-sample}} - \delta^{18}O_{CO2\text{-standard}}$. The deviation is considered as significant when $\Delta^{13}C$ and $\Delta^{18}O$ values are higher than 0.2‰, i.e., slightly higher than the 2σ precision for $\delta^{13}C$ and $\delta^{18}O$. Additionally, in order to evaluate the potential contribution of CO_2 produced by yeast during H_3PO_4 digestion, the $\delta^{13}C_{CO2}$ and $\delta^{18}O_{CO2}$ (vs. PDB) values of the evolved CO_2 produced by pure yeast H_3PO_4 digestion were also determined (data in Tables 1 and 2).

Untreated samples

Calcite and calcite–yeast mixtures—H_3PO_4 digestion at 25°C

The $\delta^{13}C_{CO2}$ and $\delta^{18}O_{CO2}$ values for pure calcite R0 obtained by CF-IRMS are −9.82‰ ± 0.14‰ and −9.12‰ ± 0.10‰ respectively (2σ, $n = 32$) and are identical to the values obtained by the classical method of McCrea (1950) which uses cryogenic purification rather than gas chromatographic purification (Table 1). All synthetic mixtures have $\delta^{13}C_{CO2}$ values similar to pure calcite R0, even for high yeast content (Fig. 1A). In contrast, their $\delta^{18}O_{CO2}$ values deviate from that of R0 when yeast proportion is ≥50%. They increase from −8.86‰ to −6.78‰ ($\Delta^{18}O$ from 0.26‰ to 2.34‰) for yeast proportions from 50% to 95% (Fig. 1B). In addition, the

Table 1. $\delta^{13}C_{CO2}$ and $\delta^{18}O_{CO2}$ values of pure calcite, synthetic samples and pure yeast. $\Delta^{13}C$ (= $\delta^{13}C_{CO2\text{-sample}} - \delta^{13}C_{CO2\text{-standard}}$) and $\Delta^{18}O$ (= $\delta^{18}O_{CO2\text{-sample}} - \delta^{18}O_{CO2\text{-standard}}$) correspond to offsets from the isotopic values of pure calcite.

Sample			Yeast/sol.	Time	*n*	*n*	*n*	$\delta^{13}C$	$\Delta^{13}C$	± 2σ	$\delta^{18}O$	$\Delta^{18}O$	± 2σ	Calcite	± 2σ	Mass loss
Calcite	Yeast	Method	(g/L)	(h)	AP	GB	Cryo	(‰)	(‰)	(‰)	(‰)	(‰)	(‰)	(wt%)	(wt%)	(wt%)
100%	0%	Untreated			32			−9.82	—	0.14	−9.12	—	0.10	99.9	5.1	
90%	10%	Untreated			8			−9.81	0.01	0.14	−8.97	0.15	0.13	93.8	5.8	
75%	25%	Untreated			8			−9.83	−0.01	0.15	−8.95	0.17	0.14	77.9	6.3	
50%	50%	Untreated			8			−9.84	−0.02	0.08	−8.86	**0.26**	0.11	50.4	7.3	
25%	75%	Untreated			8			−9.86	−0.04	0.11	−8.56	**0.56**	**0.32**	24.6	2.3	
10%	90%	Untreated			11			−9.86	−0.04	0.21	−7.70	**1.42**	**0.85**	9.2	1.6	
5%	95%	Untreated			7			−9.78	0.04	0.11	−6.78	**2.34**	**0.63**	4.2	0.7	
0%	100%	Untreated				4		−26.01	**−16.19**	**2.29**	−6.36	**2.76**	**1.62**	0.0033*	0.0026	
100%	0%	Untreated					2	−9.82		0.02	−9.12		0.01			
90%	10%	Untreated					3	−9.81	0.00	0.04	−9.06	0.06	0.03			
75%	25%	Untreated					3	−9.83	−0.01	0.02	−9.09	0.04	0.07			
25%	75%	Untreated					3	−9.85	−0.03	0.03	−9.01	0.11	0.01			
10%	90%	Untreated					1	−9.86	−0.05		−8.93	0.19				
100%	0%	H_2O_2-A	0	24	4			−9.90	−0.08	0.09	−9.18	−0.06	0.12	98.8	2.7	32.7
90%	10%	H_2O_2-A	1.7	24	4			−9.90	−0.08	0.09	−8.99	0.13	0.08	95.4	3.5	53.7
75%	25%	H_2O_2-A	2.5	24	4			−9.88	−0.06	0.08	−8.95	0.17	0.10	96.4	3.2	38.8
50%	50%	H_2O_2-A	7.5	24	4			−9.87	−0.05	0.12	−8.71	**0.41**	0.17	67.1	3.2	62.0
25%	75%	H_2O_2-A	7.5	24	4			−9.88	−0.06	0.17	−7.94	**1.18**	**0.31**	32.3	3.1	50.8
10%	90%	H_2O_2-A	16.9	24	4			−9.86	−0.04	0.15	−3.42	**5.70**	**1.26**	8.5	0.7	56.2
100%	0%	H_2O_2-B	0	72				Nm	—	—	Nm	—	—	Nm	—	75.3
90%	10%	H_2O_2-B	0.1	72	4			−9.89	−0.07	0.13	−9.14	−0.02	0.08	96.6	9.0	48.8
75%	25%	H_2O_2-B	0.3	72	4			−9.88	−0.06	0.14	−9.03	0.09	0.06	94.3	4.1	58.6
50%	50%	H_2O_2-B	0.6	72	4			−9.86	−0.04	0.07	−8.99	0.13	0.08	95.7	6.9	68.5
25%	75%	H_2O_2-B	0.9	72	4			−9.88	−0.06	0.11	−8.84	**0.28**	0.08	82.6	10.5	84.9
10%	90%	H_2O_2-B	1.6	72	4			−9.76	0.06	0.29	−6.00	**3.12**	**0.31**	19.6	4.5	84.9
5%	95%	H_2O_2-B	2.9	72	4			−12.68	**−2.86**	**1.50**	−6.71	**2.41**	**0.96**	1.9	1.5	56.2
0%	100%	H_2O_2-A	18.8	24		1		−40.53	**−30.71**	—	2.33	**11.45**	—	0.089*	—	87.5
100%	0%	NaOCl-A	0	24	4			−9.81	0.01	0.18	−9.06	0.06	0.08	99.2	1.8	36.0
90%	10%	NaOCl-A	0.6	24	4			−9.84	−0.02	0.09	−8.95	0.17	0.02	102.1	2.6	39.9
75%	25%	NaOCl-A	1.3	24	4			−9.84	−0.02	0.18	−8.93	0.19	0.05	100.9	8.0	53.3
50%	50%	NaOCl-A	2.1	24	4			−9.83	−0.01	0.12	−8.97	0.15	0.15	100.8	5.9	71.4
25%	75%	NaOCl-A	4.3	24	4			−9.88	−0.06	0.20	−8.86	**0.26**	0.14	93.5	16.0	87.1
10%	90%	NaOCl-A	6.8	24	4			−9.88	−0.06	0.05	−8.80	**0.32**	**0.33**	58.3	14.4	90.9
100%	0%	NaOCl-B	0	48	4			−9.83	−0.01	0.12	−9.07	0.05	0.14	98.2	3.5	15.8
25%	75%	NaOCl-B	2.1	48	4			−9.89	−0.07	0.11	−9.02	0.10	0.10	99.3	5.3	82.8
10%	90%	NaOCl-B	3.8	48	4			−9.86	−0.04	0.14	−8.95	0.17	0.08	81.4	7.5	92.7
5%	95%	NaOCl-B	4.8	48	4			−9.83	−0.01	0.09	−9.00	0.12	0.17	83.2	6.1	95.4
0%	100%	NaOCl-A	7.5	24				Nm	—	—	Nm	—	—	Nm	—	100.0

Yeast (mg)													
100%	0%	Plasma		1	4	−9.84	−0.02	0.10	−9.10	0.02	0.06	99.5	0.5	1.0
100%	0%	Plasma		5	4	−9.85	−0.03	0.07	−9.12	0.00	0.14	102.6	2.1	3.3
90%	10%	Plasma	2	1	4	−9.83	−0.01	0.03	−9.10	0.02	0.08	95.9	5.9	10.4
75%	25%	Plasma	5	1	4	−9.87	−0.05	0.13	−9.09	0.03	0.08	92.9	5.9	22.0
50%	50%	Plasma	15	2	4	−9.88	−0.06	0.04	−9.12	0.00	0.05	87.9	6.9	48.6
25%	75%	Plasma	45	3	4	−9.86	−0.04	0.10	−9.17	−0.05	0.07	80.8	12.7	71.4
10%	90%	Plasma	135	5	4	−9.95	−0.13	0.12	−9.29	−0.17	0.15	48.9	12.8	84.1
5%	95%	Plasma	285	8	4	−9.92	−0.10	0.05	−9.25	−0.13	0.09	39.3	8.1	89.8
0%	100%	Plasma	750	5		Nm	—		Nm	—	—	Nm	—	89.0

Notes: The bold values indicate an offset equal to or higher than 0.2‰. Standard deviations values equals to or higher than 0.3‰ are also in bold.
n is the number of tubes measured by AP (Analytical Precision 2003 CF-IRMS), GB (Gas Bench coupled with a Delta plus XP CF-IRMS) or Cryo (off-line cryogenic method and dual inlet IRMS) (*see* Materials and methods).
Nm: Not measured.
The letters A and B added after H_2O_2 and NaOCl distinguish 2 batches with different conditions of H_2O_2 and NaOCl treatments (*see* Materials and methods).
*indicates μ_{yeast} value for untreated yeast and yeast treated by H_2O_2 (*see* Discussion).

external reproducibility of $\delta^{18}O_{CO2}$ measurements worsens as $\Delta^{18}O$ increases, with values up to ±0.85‰ (2σ).

Dolomite and dolomite–yeast mixtures—H_3PO_4 digestion at 80°C

The $\delta^{13}C_{CO2}$ and $\delta^{18}O_{CO2}$ values for pure dolomite INYO analyzed by classical method are −0.47‰ ± 0.01‰ and 1.91‰ ± 0.03‰ respectively (2σ, $n = 9$; Table 2). The $\delta^{13}C_{CO2}$ values for synthetic mixtures with yeast proportions ≥75% are shifted to more negative values, with $\Delta^{13}C$ reaching −0.66‰ for the sample with 95% of yeast. Surprisingly $\delta^{18}O_{CO2}$ values do not show any deviation, within uncertainty, regardless of the dolomite-yeast mixture proportion (Table 2 and Fig. 2).

Pure yeast

The $\delta^{13}C_{CO2}$ value of pure yeast determined by sealed tube combustion method is −24.52‰ ± 0.12‰ (2σ, $n = 3$). The $\delta^{13}C_{CO2}$ and $\delta^{18}O_{CO2}$ values measured by CF-IRMS for CO_2 released by yeast reacted with H_3PO_4 at 25°C are −26.01‰ ± 2.29‰ and −6.36‰ ± 1.62‰, respectively (Table 1). At 80°C, the values are of −38.39‰ ± 2.60‰ and −12.19‰ ± 2.48‰, respectively (Table 2). The precisions are particularly low possibly owing to impurities and/or non-reproducibility of the yeast digestion by H_3PO_4.

Samples treated with H_2O_2

Calcite and calcite–yeast mixtures—H_3PO_4 digestion at 25°C

Pure calcite samples were partially dissolved by H_2O_2 treatment. They lost 32.7% of their mass in the H_2O_2-A protocol and 75.3% in the longer H_2O_2-B protocol (Table 1). In spite of calcite dissolution, neither $\delta^{13}C_{CO2}$ nor $\delta^{18}O_{CO2}$ were modified. Treated synthetic mixtures show both partial dissolution of calcite and incomplete organic matter removal. Calcite dissolution can be evidenced in a diagram of mass loss vs. initial yeast proportion (Fig. 3B). Samples with yeast proportion lower than 75% plot above the 1:1 line, indicating that the mass loss was higher than initial yeast proportion, and hence that calcite must have been dissolved. For 95% of yeast, only 1.9% of calcite instead of 5% remained after treatment by the H_2O_2-B protocol, demonstrating drastic calcite dissolution. Incomplete organic matter removal is evidenced only in mixtures with initial yeast proportions ≥50%, as illustrated by their calcite content lower than the theoretically expected 100% (Table 1 and Fig. 3A). Organic matter removal was improved by the longer treatment of H_2O_2-B protocol but remained incomplete for samples with initial yeast proportions ≥75%. The $\delta^{13}C_{CO2}$ values of sample mixtures are indistinguishable from pure calcite R0, except for 95% of yeast with a $\Delta^{13}C$ value of −2.86‰ (Table 1 and Fig. 1A). For O isotopes, samples for which organic matter was quantitatively removed by H_2O_2 treatment have $\delta^{18}O_{CO2}$ values similar to untreated calcite R0. In contrast, samples still containing organic matter after reaction

Table 2. $\delta^{13}C_{CO2}$ and $\delta^{18}O_{CO2}$ values of pure dolomite, synthetic samples and pure yeast. $\Delta^{13}C$ (= $\delta^{13}C_{CO2\text{-sample}} - \delta^{13}C_{CO2\text{-standard}}$) and $\Delta^{18}O$ (= $\delta^{18}O_{CO2\text{-sample}} - \delta^{18}O_{CO2\text{-standard}}$) correspond to offsets from the isotopic values of pure dolomite.

Sample			Yeast/sol.	Time	n	n	n	$\delta^{13}C$	$\Delta^{13}C$	$\pm 2\sigma$	$\delta^{18}O_{CO2}$	$\Delta^{18}O$	$\pm 2\sigma$	Dolomite	$\pm 2\sigma$	Mass loss
Dolomite	Yeast	Method	(g/L)	(h)	AP	GB	Cryo	(‰)	(‰)	(‰)	(‰)	(‰)	(‰)	(wt%)	(wt%)	(wt%)
100%	0%	Untreated					9	−0.47	—	0.01	1.91	—	0.03			
90%	10%	Untreated			2	2		−0.46	0.01	0.15	1.89	−0.03	0.16	92.6	11.1	
75%	25%	Untreated			2	2		−0.44	0.03	0.12	1.88	−0.03	0.06	74.4	16.7	
50%	50%	Untreated			2	2		−0.51	−0.04	0.04	1.87	−0.04	0.15	54.9	10.3	
25%	75%	Untreated			2	2		−0.57	−0.10	0.10	1.88	−0.03	0.19	25.2	8.4	
10%	90%	Untreated			2	4		−0.76	**−0.29**	0.19	1.91	0.00	0.21	9.8	1.6	
5%	95%	Untreated			2	4		−1.13	**−0.66**	**0.39**	1.81	−0.10	0.18	4.5	1.3	
0%	100%	Untreated				7		−38.39	**−37.92**	**2.60**	−12.19	**−14.11**	**2.48**	0.057*	0.023	
100%	0%	H_2O_2-B		72		4		−0.48	−0.01	0.23	1.93	0.01	0.14	87.0	5.8	41.4
90%	10%	H_2O_2-B	0.1	72		4		−0.51	−0.04	0.22	1.98	0.07	0.08	92.9	6.5	43.9
75%	25%	H_2O_2-B	0.2	72		4		−0.52	−0.05	0.13	1.94	0.03	0.07	81.0	17.2	48.5
50%	50%	H_2O_2-B	0.6	72		4		−0.54	−0.07	0.16	1.98	0.07	0.00	85.1	10.8	50.3
25%	75%	H_2O_2-B	1.8	72		4		−0.50	−0.03	0.22	2.01	0.10	0.14	30.3	6.4	53.8
10%	90%	H_2O_2-B	2.7	72		4		−0.80	**−0.33**	**0.34**	1.90	−0.01	0.11	15.5	1.3	51.9
5%	95%	H_2O_2-B	2.9	72		4		−7.01	**−6.54**	**0.93**	1.56	**−0.36**	0.18	8.9	1.0	51.4
0%	100%	H_2O_2-B	3.0	72		1		−43.90	**−43.43**	—	−2.09	**−4.01**	—	1.5*	—	56.9
100%	0%	NaOCl-B		48		4		−0.46	0.01	0.10	1.94	0.03	0.08	91.5	10.2	6.4
90%	10%	NaOCl-B	0.1	48		4		−0.39	0.08	0.12	1.91	0.00	0.09	90.5	13.1	45.8
75%	25%	NaOCl-B	0.3	48		4		−0.44	0.03	0.12	1.86	−0.05	0.12	92.1	15.6	38.3
50%	50%	NaOCl-B	1.0	48		4		−0.42	0.05	0.19	1.85	−0.06	0.06	91.2	13.4	58.3
25%	75%	NaOCl-B	3.0	48		4		−0.43	0.04	0.17	1.86	−0.05	0.15	93.4	6.7	80.1
10%	90%	NaOCl-B	3.9	48		4		−0.46	0.01	0.19	1.83	−0.08	0.17	87.9	7.9	90.6
5%	95%	NaOCl-B	4.8	48		4		−0.50	−0.03	0.18	1.85	−0.06	0.26	77.3	3.9	93.6
0%	100%	NaOCl-B	5.0	48				Nm	—	—	Nm	—	—	Nm	—	98.7
			Yeast (mg)													
100%	0%	Plasma		1	2	2		−0.47	0.00	0.11	1.89	−0.02	0.11	99.8	5.1	0.9
90%	10%	Plasma	2	1	2	2		−0.50	−0.03	0.10	1.84	−0.07	0.09	94.5	4.7	9.5
75%	25%	Plasma	5	1	2	2		−0.52	−0.05	0.03	1.87	−0.04	0.13	86.2	7.5	20.1
50%	50%	Plasma	15	2	2	2		−0.52	−0.05	0.13	1.90	−0.01	0.13	86.8	6.4	45.5
25%	75%	Plasma	45	3	2	2		−0.61	−0.14	0.04	1.80	−0.11	0.21	78.6	21.6	69.0
10%	90%	Plasma	135	5	2	2		−0.72	**−0.25**	0.11	1.66	**−0.25**	0.20	55.7	6.2	84.6
5%	95%	Plasma	285	8	2	2		−0.93	**−0.46**	0.19	1.46	**−0.45**	0.28	38.5	14.3	89.0
0%	100%	Plasma	750	5		2		−26.22	**−25.75**	**1.58**	−25.63	**−27.55**	**1.63**	0.32*	0.055	89.0
10%	90%	Plasma-bis	135	10		4		−0.62	−0.15	**0.31**	1.92	0.01	0.13	64.4	23.6	86.0
5%	95%	Plasma-bis	285	15		4		−0.66	−0.19	**0.31**	1.70	**−0.21**	**0.56**	39.8	27.1	90.4

Notes: The bold values indicate an offset equal to or higher than 0.2‰. Standard deviations values equal to or higher than 0.3‰ are also in bold.
n is the number of tubes measured by AP (Analytical Precision 2003 CF-IRMS), GB (Gas Bench coupled with a Delta plus XP CF-IRMS) or Cryo (off-line cryogenic method and dual inlet IRMS) (*see* Materials and methods).
Nm: Not measured.
*indicates μ_{yeast} value for untreated yeast and yeast treated by H_2O_2 (*see* Discussion).

with H_2O_2 present large deviations in $\delta^{18}O_{CO2}$ up to +5.70 ‰ with a poor analytical precision (Table 1 and Fig. 1B).

Dolomite and dolomite–yeast mixtures—H_3PO_4 Digestion at 80°C

For dolomite, only the more efficient H_2O_2-B protocol was used. For pure dolomite samples, only minor dissolution occurred (Fig. 4A; Table 2) and neither $\delta^{13}C_{CO2}$ nor $\delta^{18}O_{CO2}$ were affected by H_2O_2 treatment (as for pure calcite samples) (Fig. 2 and Table 2). For dolomite-yeast mixtures, organic matter removal was not complete for samples containing more than 50% of yeast (Fig. 4A) and dolomite was partly dissolved, as illustrated by samples plotting above the line 1:1 in Fig. 4B. The $\delta^{13}C_{CO2}$ values of sample mixtures are indistinguishable from untreated pure dolomite INYO until a yeast proportion of 90%, which shows a minor 0.33‰ shift. For 95% of yeast, $\delta^{13}C_{CO2}$ shifts to strongly lighter values ($\Delta^{13}C = -6.54$‰; Table 2 and Fig. 2A). A similar pattern is observed for $\delta^{18}O_{CO2}$, but with a much smaller shift at 95% only ($\Delta^{18}O = -0.36$‰; Table 2 and Fig. 2B).

Pure yeast residues—H_3PO_4 digestion at 25 and 80°C

The yeast residue (~100 mg) treated by the H_2O_2-A protocol and reacted with H_3PO_4 at 25°C had $\delta^{13}C_{CO2}$ and $\delta^{18}O_{CO2}$ values of −40.53‰ and 2.33‰, respectively (Table 1). The external precision on these measurements was not determined because the residue recovered after treatment was too small for more than one analysis. The yeast residue (~90 mg) treated by the H_2O_2-B protocol and reacted with H_3PO_4 at 80°C had $\delta^{13}C_{CO2}$ and $\delta^{18}O_{CO2}$ values of −43.90‰ and −2.09‰ respectively (Table 2). In order to further investigate optimum conditions for organic matter removal, 300 mg of pure yeast were reacted with three different volumes of H_2O_2 (50, 100, 200 ml) and for three reaction times (24, 48, and 120 h). The results showed a limited range of mass loss, between 47.1% and 55.4%, for the different conditions. However, there was a slight increase of yeast loss with increasing reaction time (Table 3) but none with increasing volume of H_2O_2 for a given reaction time. Increasing further the reaction time (216 h for 600 mg of yeast and 50 ml of H_2O_2) improved the mass loss but only up to 73.7% (Table 3).

Samples treated with NaOCl

Calcite and calcite–yeast mixtures—H_3PO_4 digestion at 25°C

Like for H_2O_2, two different NaOCl protocols were tested. Pure calcite samples were partially dissolved by both treatments, losing up to 36% of their mass (Table 1 and Fig. 3B). Neither $\delta^{13}C_{CO2}$ nor $\delta^{18}O_{CO2}$ were modified (Table 1 and Fig. 1). For synthetic mixtures, calcite was partially dissolved by the NaOCl treatment, as shown by higher mass loss than

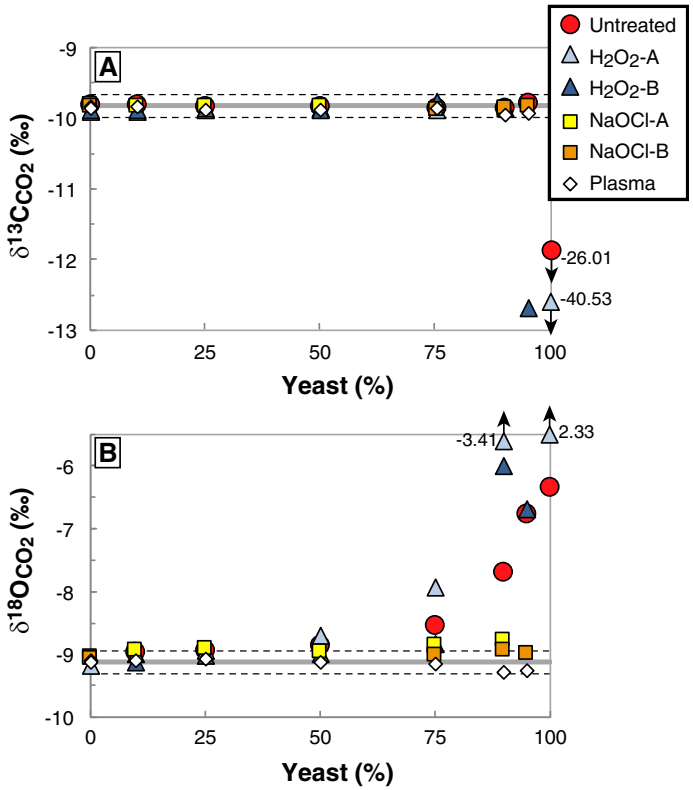

Fig 1. $\delta^{13}C_{CO2}$ (**A**) and $\delta^{18}O_{CO2}$ (**B**) values of the calcite mixture samples as a function of the yeast proportion (%). The solid gray lines represent the average isotope compositions of pure calcite (R0) and the dotted lines indicate a ± 0.2% range. The errors are comprised within symbols.

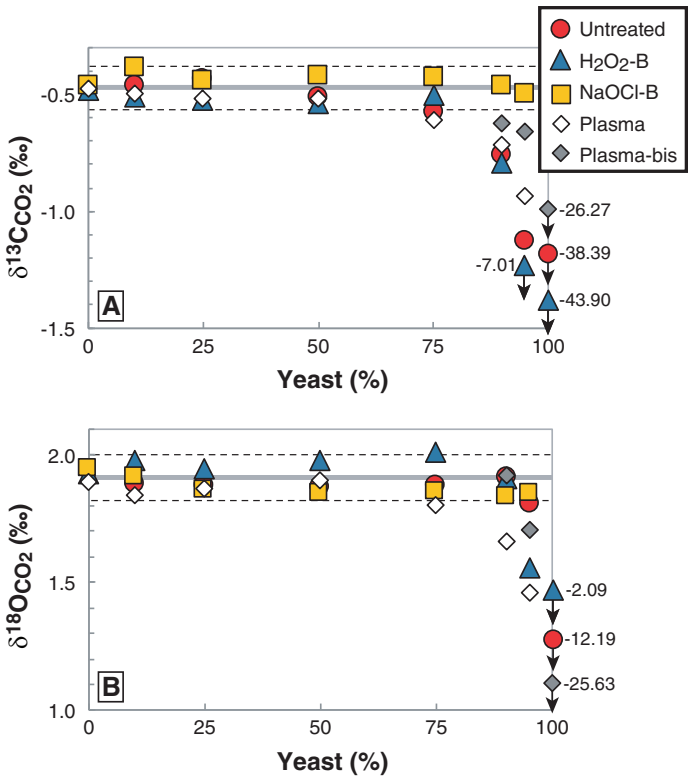

Fig 2. $\delta^{13}C_{CO2}$ (**A**) and $\delta^{18}O_{CO2}$ (**B**) values of the dolomite mixture samples as a function of the yeast proportion (%). The gray lines represent the average isotope compositions of pure dolomite (INYO) and the dotted lines indicate a ±0.1‰ range. The errors are comprised within symbols.

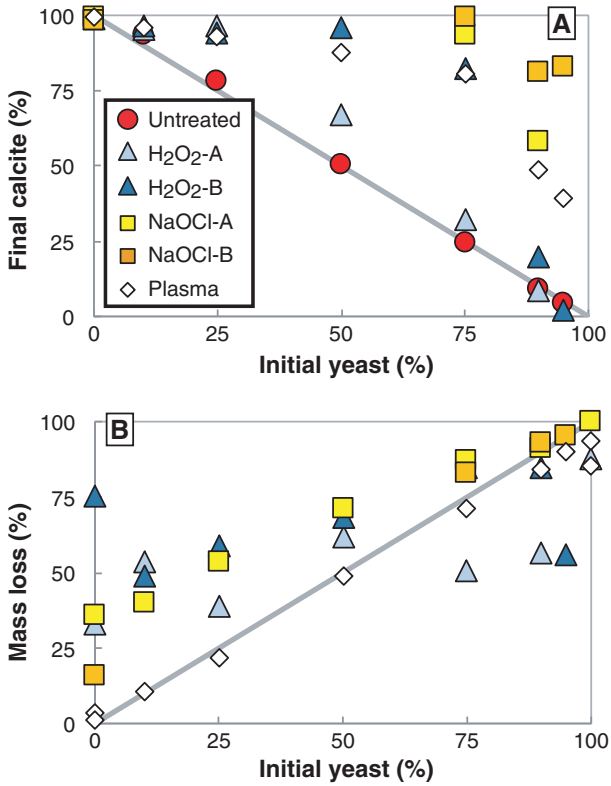

Fig 3. Efficiency of yeast removal from calcite mixture samples. (**A**) Final proportion of calcite (%) vs. initial proportion of yeast (%). As expected, untreated samples fall on the gray −1:1 line, while treated samples fall above this line, indicating yeast loss that exceeds carbonate loss. (**B**) Mass loss (%) vs. initial proportion of yeast (%). The gray 1:1 line indicates quantitative yeast loss without carbonate loss. LTA (i.e., plasma) treated samples fall on or close to this line. Samples falling above this line indicate that carbonate loss occurred in addition to yeast loss. Samples falling below this line indicate non-quantitative yeast loss.

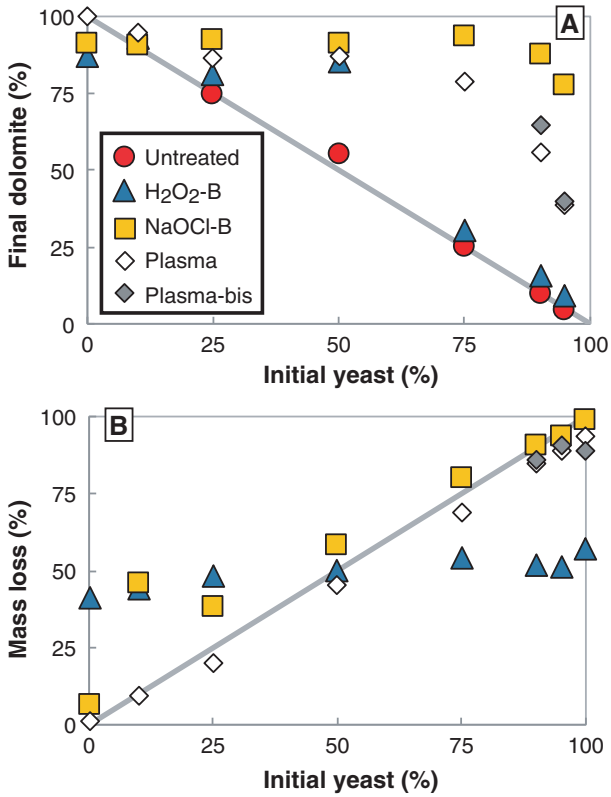

Fig 4. Efficiency of yeast removal from dolomite mixture samples: (**A**) Final proportion of dolomite (%) vs. initial proportion of yeast (%). As expected, untreated samples fall on the gray −1:1 line, while treated samples fall above this line, indicating yeast loss that exceeds carbonate loss. (**B**) Mass loss (%) vs. initial proportion of yeast (%). The gray 1:1 line indicates quantitative yeast loss without carbonate loss. LTA (i.e., plasma) treated samples fall on or close to this line. Samples falling above this line indicate that carbonate loss occurred in addition to yeast loss. Samples falling below this line indicate non-quantitative yeast loss.

expected from initial yeast proportion (i.e., most data plotted above the line 1:1 in Fig. 3B). Organic matter removal was incomplete. For the NaOCl-A protocol, samples with a yeast proportion ≥ 75% present calcite contents lower than 100% (from 93.5 to 58.3%) (Table 1 and Fig. 3A). For the longer NaOCl-B protocol, organic matter removal was improved but remained incomplete for yeast proportions ≥90%, with calcite contents in the residue ranging from 81.4% to 83.2%. In terms of isotope composition, the $\delta^{13}C_{CO2}$ values are indistinguishable from untreated pure calcite R0 irrespectively of the protocol or yeast content (Table 1 and Fig. 1A). $\delta^{18}O_{CO2}$ values were only slightly shifted in protocol A for yeast contents ≥75% (with $\Delta^{18}O$ values up to +0.32‰), while no shift was identified in protocol B (Table 1 and Fig. 1B).

Dolomite and dolomite–yeast mixtures—H_3PO_4 digestion at 80°C

Only NaOCl-B protocol was used for dolomite samples. Pure dolomite was slightly dissolved (6.4% of mass loss; Table 2 and Fig. 4B) with no modification of $\delta^{13}C_{CO2}$ and $\delta^{18}O_{CO2}$ values

Table 3. Mass loss of yeast reacted with H_2O_2 and NaOCl for different conditions of time and volume of H_2O_2 or NaOCl.

H_2O_2 (ml)	NaOCl (ml)	Yeast (mg)	Yeast/sol. (g/l)	Time (h)	Mass loss (wt%)
100		300	3.0	24	47.1
100		300	3.2	48	51.3
100		300	3.0	120	55.4
50		300	6.3	48	48.3
200		300	1.6	48	50.2
50		600	12.0	216	73.7
	30	300	10.0	24	92.6
	30	300	9.9	48	97.0
	30	300	10.6	120	93.8
	15	300	22.2	48	89.0
	70	300	4.4	48	98.2
	30	600	20.0	216	97.3

Table 4. Calculated $\delta^{13}C_{CO2}$ and $\delta^{18}O_{CO2}$ values for the CO_2 produced by calcite mixture samples during H_3PO_4 digestion, assuming CO_2 originates from yeast and calcite (*see* text for calculation). The calculated values are compared to the measured values (also displayed in Table 1).

Sample			$\omega_{calcite}$	ω_{yeast}	$\delta^{13}C_{meas}$	$\Delta^{13}C_{meas}$	$\delta^{13}C_{calcul}$	$\Delta^{13}C_{calcul}$	$\delta^{18}O_{meas}$	$\Delta^{18}O_{meas}$	$\delta^{18}O_{calcul}$	$\Delta^{18}O_{calcul}$
Calcite	Yeast	Method	(%)	(%)	(‰)	(‰)	(‰)	(‰)	(‰)	(‰)	(‰)	(‰)
100%	0%	Untreated	100.0	0.0	−9.82	—	−9.82	—	−9.12	—	−9.12	—
90%	10%	Untreated	93.8	6.2	−9.81	0.01	−9.82	0.00	−8.97	0.15	−9.12	0.00
75%	25%	Untreated	77.9	22.1	−9.83	−0.01	−9.82	0.00	−8.95	0.17	−9.12	0.00
50%	50%	Untreated	50.4	49.6	−9.84	−0.02	−9.82	0.00	−8.86	**0.26**	−9.12	0.00
25%	75%	Untreated	24.6	75.4	−9.86	−0.04	−9.82	0.00	−8.56	**0.56**	−9.12	0.00
10%	90%	Untreated	9.2	90.8	−9.86	−0.04	−9.83	−0.01	−7.70	**1.42**	−9.12	0.00
5%	95%	Untreated	4.2	95.8	−9.78	0.04	−9.83	−0.01	−6.78	**2.34**	−9.12	0.00
0%	100%	Untreated	0.0	100.0	−26.01	—	−26.01	—	−6.36	—	−6.36	—
100%	0%	H_2O_2-A	100.0	0.0	−9.90	−0.08	−9.82	—	−9.18	−0.06	−9.12	—
90%	10%	H_2O_2-A	95.4	4.6	−9.90	−0.08	−9.82	0.00	−8.99	0.13	−9.12	0.00
75%	25%	H_2O_2-A	96.4	3.6	−9.88	−0.06	−9.82	0.00	−8.95	0.17	−9.12	0.00
50%	50%	H_2O_2-A	67.1	32.9	−9.87	−0.05	−9.83	−0.01	−8.71	**0.41**	−9.11	0.01
25%	75%	H_2O_2-A	32.3	67.7	−9.88	−0.06	−9.88	−0.06	−7.94	**1.18**	−9.10	0.02
10%	90%	H_2O_2-A	8.5	91.5	−9.86	−0.04	−10.11	**−0.29**	−3.42	**5.70**	−9.01	0.11
90%	10%	H_2O_2-B	96.6	3.4	−9.89	−0.07	−9.82	0.00	−9.14	−0.02	−9.12	0.00
75%	25%	H_2O_2-B	94.3	5.7	−9.88	−0.06	−9.82	0.00	−9.03	0.09	−9.12	0.00
50%	50%	H_2O_2-B	95.7	4.3	−9.86	−0.04	−9.82	0.00	−8.99	0.13	−9.12	0.00
25%	75%	H_2O_2-B	82.6	17.4	−9.88	−0.06	−9.83	−0.01	−8.84	**0.28**	−9.12	0.00
10%	90%	H_2O_2-B	19.6	80.4	−9.76	0.06	−9.93	−0.11	−6.00	**3.12**	−9.08	0.04
5%	95%	H_2O_2-B	1.9	98.1	−12.68	**−2.86**	−11.18	**−1.36**	−6.71	**2.41**	−8.61	**0.51**
0%	100%	H_2O_2-A	0.0	100.0	−40.53	—	−40.53	—	2.33	—	2.33	—

Table 5. Calculated $\delta^{13}C_{CO2}$ and $\delta^{18}O_{CO2}$ values of the CO_2 produced by dolomite mixture samples during H_3PO_4 digestion, assuming CO_2 originates from yeast and dolomite (*see* text for calculation). The calculated values are compared to the measured values (also displayed in Table 2).

Sample			$\omega_{dolomite}$	ω_{yeast}	$\delta^{13}C_{meas}$	$\Delta^{13}C_{meas}$	$\delta^{13}C_{calcul}$	$\Delta^{13}C_{calcul}$	$\delta^{18}O_{meas}$	$\Delta^{18}O_{meas}$	$\delta^{18}O_{calcul}$	$\Delta^{18}O_{calcul}$
Dolomite	**Yeast**	**Method**	**(wt%)**	**(wt%)**	**(‰)**	**(‰)**	**(‰)**	**(‰)**	**(‰)**	**(‰)**	**(‰)**	**(‰)**
100%	0%	Untreated	100.0	0	−0.47	—	−0.47	—	1.91	—	1.91	—
90%	10%	Untreated	92.6	7.4	−0.46	0.01	−0.47	0.00	1.89	−0.03	1.91	0.00
75%	25%	Untreated	74.4	25.6	−0.44	0.03	−0.48	−0.01	1.88	−0.03	1.91	0.00
50%	50%	Untreated	54.9	45.1	−0.51	−0.04	−0.49	−0.02	1.87	−0.04	1.90	−0.01
25%	75%	Untreated	25.2	74.8	−0.57	−0.10	−0.53	−0.06	1.88	−0.03	1.89	−0.02
10%	90%	Untreated	9.8	90.2	−0.76	**−0.29**	−0.67	**−0.20**	1.91	0.00	1.84	−0.07
5%	95%	Untreated	4.5	95.5	−1.13	**−0.66**	−0.92	**−0.45**	1.81	−0.10	1.74	−0.17
0%	100%	Untreated	0.0	100.0	−38.39	—	−38.39	—	−12.19	—	−12.19	—
100%	0%	H_2O_2-B	100.0	0.0	−0.48	−0.01	−0.47	—	1.93	0.01	1.91	—
90%	10%	H_2O_2-B	*92.9*	*7.1*	−0.51	−0.04	−0.52	−0.05	1.98	0.07	1.91	0.00
75%	25%	H_2O_2-B	*81.0*	*19.0*	−0.52	−0.05	−0.63	−0.16	1.94	0.03	1.90	−0.01
50%	50%	H_2O_2-B	*85.1*	*14.9*	−0.54	−0.07	−0.59	−0.12	1.98	0.07	1.90	−0.01
25%	75%	H_2O_2-B	*30.3*	*69.7*	−0.50	−0.03	−1.95	**−1.48**	2.01	0.10	1.77	−0.14
10%	90%	H_2O_2-B	*15.5*	*84.5*	−0.80	**−0.33**	−3.82	**−3.35**	1.90	−0.01	1.60	**−0.31**
5%	95%	H_2O_2-B	*8.9*	*91.1*	−7.01	**−6.54**	−6.37	**−5.90**	1.56	**−0.36**	1.37	**−0.54**
0%	100%	H_2O_2-B	0.0	100.0	−43.90	—	−43.90	—	−2.09	—	−2.09	—
100%	0%	Plasma	100	0	−0.47	0.00	−0.47	—	1.89	−0.02	1.91	—
90%	10%	Plasma	*94.5*	*5.5*	−0.50	−0.03	−0.47	0.00	1.84	−0.07	1.91	−0.01
75%	25%	Plasma	*86.2*	*13.8*	−0.52	−0.05	−0.48	−0.01	1.87	−0.04	1.90	−0.01
50%	50%	Plasma	*86.8*	*13.2*	−0.52	−0.05	−0.48	−0.01	1.90	−0.01	1.90	−0.01
25%	75%	Plasma	*78.6*	*21.4*	−0.61	−0.14	−0.49	−0.02	1.80	−0.11	1.89	−0.02
10%	90%	Plasma	*55.7*	*44.3*	−0.72	**−0.25**	−0.54	−0.07	1.66	**−0.25**	1.84	−0.07
5%	95%	Plasma	*38.5*	*61.5*	−0.93	**−0.46**	−0.60	−0.13	1.46	**−0.45**	1.77	−0.14
10%	90%	Plasma-bis	*64.4*	*35.6*	−0.62	−0.15	−0.52	−0.05	1.92	0.01	1.86	−0.05
5%	95%	Plasma-bis	*39.8*	*60.2*	−0.66	−0.19	−0.59	−0.12	1.70	**−0.21**	1.78	−0.13
0%	100%	Plasma	0	100	−26.22	—	−26.22	—	−25.63	—	−25.63	—

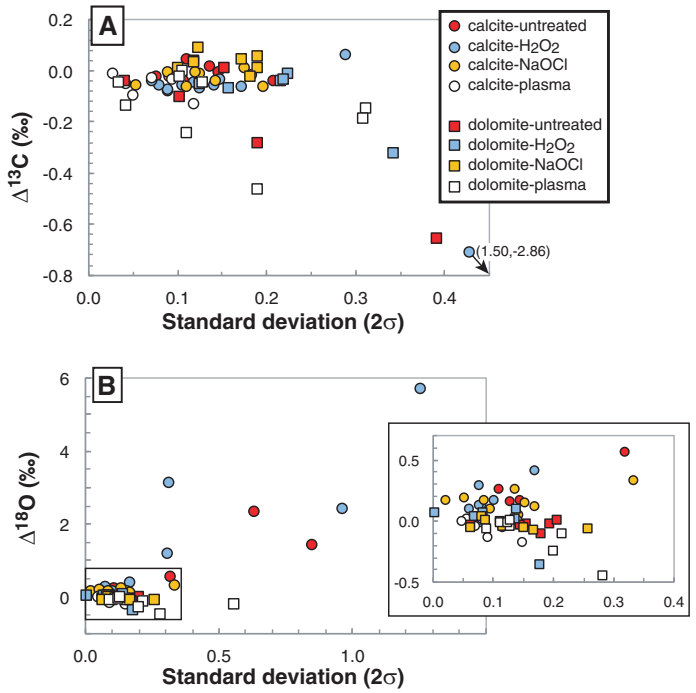

Fig 5. Isotopic shifts $\Delta^{13}C$ (**A**) and $\Delta^{18}O$ (**B**) of mixture samples as a function of the external reproducibility on isotope measurements (2σ).

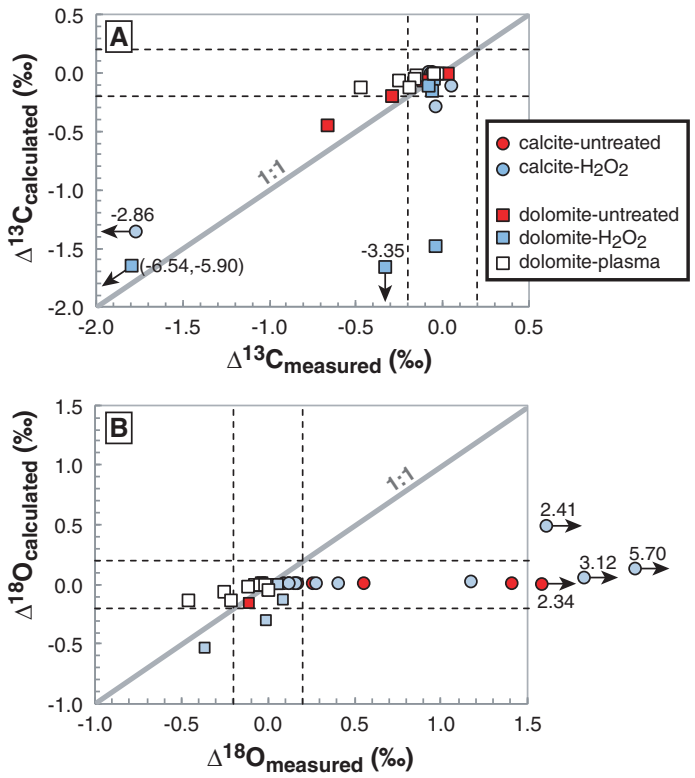

Fig 6. Comparison between the isotope shifts measured for mixture samples and those calculated assuming a CO_2 contribution from yeast. The gray line represents the 1:1 line where the points should align in case of a CO_2 contribution from yeast.

(Table 2 and Fig. 2). For synthetic mixtures, yeast was quantitatively removed by NaOCl, except for proportions of 90 and 95% (Table 2 and Fig. 4A) in which a small fraction of organic matter was left (12% and 23%, respectively). The NaOCl-B treatment also dissolved part of the dolomite, as evidenced by the data plotting above the line 1:1 in Fig. 4B. The $\delta^{13}C_{CO2}$ and $\delta^{18}O_{CO2}$ values of all treated mixtures were indistinguishable from untreated dolomite INYO (Table 2 and Fig. 2).

Pure yeast residues—H_3PO_4 digestion at 25°C and 80°C

The amounts of residual yeast samples treated with NaOCl were too low to be collected and prepared for isotope measurements. Nonetheless, the efficiency of organic matter removal by NaOCl has been investigated for different set of conditions, following the same procedure as for H_2O_2. Three hundred milligrams of pure yeast was reacted with three different volumes of NaOCl (15, 30, 70 ml) and three different reaction times (24, 48, and 120 h). The results showed that organic matter removal was relatively efficient, with weight losses higher than 89% (Table 3). In contrast to H_2O_2 treatment, the weight loss of yeast increased with the volume of NaOCl for a given reaction time, while the reaction time had no effect for a given volume.

Samples treated with low-temperature oxygen-plasma ashing (LTA)

Calcite and calcite-yeast mixtures—H_3PO_4 digestion at 25°C

The mass and isotope composition of pure calcite samples were not affected by LTA treatment (Table 1 and Figs. 1 and 3B). For synthetic mixtures, Fig. 3B illustrates no calcite loss with LTA treatment since all data plot either on or slightly below the 1:1 line. Organic matter removal was quantitative only in samples with less than 25% of yeast. For higher proportions, the calcite content ranged from 92.9% to 39.3% after LTA treatment (implying a residual organic matter content of 7.1–60.7%) (Table 1 and Fig. 3A). The $\delta^{13}C_{CO2}$ and $\delta^{18}O_{CO2}$ values for all synthetic mixtures are indistinguishable from those of untreated calcite R0, within uncertainty (Table 1 and Fig. 1).

Dolomite and dolomite–yeast mixtures—H_3PO_4 digestion at 80°C

The mass and isotope composition of pure dolomite samples were not affected by LTA treatment (Table 2 and Figs. 2 and 4B). For dolomite mixtures, no loss of dolomite was induced by LTA treatments since all data plot on the 1:1 line or slightly below in Fig. 4B. Similarly to calcite mixtures, organic matter removal was quantitative only in samples with less than 25% of yeast. Above this proportion, dolomite contents ranged from 86.2% to 38.5% (Table 2 and Fig. 4A) indicating the presence of residual organic matter after treatment. The $\delta^{13}C_{CO2}$ and $\delta^{18}O_{CO2}$ values of samples with yeast proportions ≥75% are slightly shifted, with $\Delta^{13}C$ and $\Delta^{18}O$ values down to −0.46‰ and −0.45‰ for the sample with 95% of yeast (Table 2 and Fig. 2). The residues of samples with yeast proportions higher than 75% were thus treated again

for 5 and 7 additional hours, respectively (they are labeled "plasma-bis" in Table 2). Although organic matter was still not completely removed, the C and O isotope data were improved, with deviations lower than −0.21‰ relative to pure dolomite (Table 2).

Pure yeast residues—H_3PO_4 digestion at 25 and 80°C

After 5 h of treatment by LTA, only ~80 mg of residue were collected from the 750 mg of pure yeast initially introduced, demonstrating that 90% of the yeast was removed. Since LTA treated mixtures reacted with H_3PO_4 at a temperature of 25°C do not present any specific isotope shift, this residue was used only for measuring the isotope composition of the CO_2 produced during an acid digestion at 80°C. The residue was divided in two subsamples of ~25 and 50 mg and reacted with H_3PO_4 at 80°C. The average $\delta^{13}C_{CO2}$ and $\delta^{18}O_{CO2}$ values were −26.22‰ ± 1.58‰ and −25.63‰ ± 1.63‰, respectively (Table 2).

Discussion

Origin of the isotope shifts

In earlier studies, the main cause suspected to have induced shifts in $\delta^{13}C_{CO2}$ and $\delta^{18}O_{CO2}$ of carbonates associated with organic matter is the production of CO_2 and/or other molecular gaseous species derived from organic matter and/or impure H_3PO_4 (Bowen 1966; Epstein et al. 1951, 1953; Falster et al. 2018; Oehlerich et al. 2013; Weber et al. 1976) and their subsequent incomplete separation prior to gas introduction in the mass spectrometer. Chromium oxide or H_2O_2 was hence added to H_3PO_4 to oxidize any organic contaminants (McCrea 1950). However, these additives may also have oxidized the sample organic matter, leading to erroneous data (Sarkar et al. 1990). This was solved using H_3PO_4 of purer quality, without chromium oxide or H_2O_2. Several studies have then shown that organic matter removal was no more required for samples with low organic content (e.g., Wierzbowski 2007). However, when the weight ratio of immature organic matter vs. carbonate is high (i.e., >25%), the present results show that this is not the case, in good agreement with three previous studies (Oehlerich et al. 2013; Lebeau et al. 2014; Falster et al. 2018). The observed shifts in $\delta^{13}C_{CO2}$ and $\delta^{18}O_{CO2}$ (both negative) were previously attributed to a contribution of CO_2 produced by organic matter during H_3PO_4 digestion (Oehlerich et al. 2013; Lebeau et al. 2014; Falster et al. 2018). Because we measured $\delta^{13}C_{CO2}$ and $\delta^{18}O_{CO2}$ values of CO_2 produced by pure yeast reaction with H_3PO_4, we can explore this hypothesis using the following isotope mass balance equation:

$$\delta^{13}C_{mixture} = \left(\omega_{yeast} \times \mu_{yeast} \times \delta^{13}C_{yeast} + \omega_{carbonate} \times \delta^{13}C_{carbonate}\right) / \left(\omega_{yeast} \times \mu_{yeast} + \omega_{carbonate}\right) \quad (1)$$

where ω is the weight proportion (wt%) of yeast or carbonate in the sample and μ_{yeast} is the weight proportion of CO_2 produced by yeast during acid digestion expressed in equivalent weight proportion of $CaCO_3$ in yeast: μ_{yeast} is 0.0033% ± 0.0026% (2σ) at 25°C (Table 1) and 0.057% ± 0.023% (2σ) at 80°C (Table 2). Using Eq. 1 and the isotopic values of the CO_2 produced by yeast reacted with H_3PO_4, the expected $\delta^{13}C_{CO2}$ and $\delta^{18}O_{CO2}$ values of mixture samples can be calculated and compared to the measured values (Tables 4 and 5).

For untreated calcite–yeast mixtures, the calculated and measured C and O isotope compositions of the CO_2 produced after H_3PO_4 digestion do not match (Table 4). The calculated results show that the amount of CO_2 generated by yeast reaction at 25°C is too small to induce a shift in $\delta^{13}C_{CO2}$ and $\delta^{18}O_{CO2}$. While this is indeed the case for $\delta^{13}C_{CO2}$, it is not true for $\delta^{18}O_{CO2}$, which presents a positive shift for samples containing more than 25% of yeast (Table 4 and Fig. 6). This implies that other molecules were likely produced during yeast digestion by H_3PO_4 at 25°C. The modification of $\delta^{18}O_{CO2}$ value suggests that at least one of these molecules was not properly separated from CO_2 by the chromatographic columns used in this study (stainless column 0.3 m × 1/8″ x 2 mm, packed with Hayesep Q 60/80 mesh, Chrompack for the AP-2003 and fused silica Poraplot Q column 25 m × 0.32 mm for the Gas Bench). Moreover, the increase of $\delta^{18}O_{CO2}$ but constancy of $\delta^{13}C_{CO2}$ indicates that, in the ionization chamber, the poorly separated molecules must form molecules or radicals of $m/z = 46$, but not 45. Several molecules, possibly interfering on m/z from 44 to 48, have already been envisaged in previous studies: C_3H_8 (44, 45, 46), C_2H_5OH (46, 47, 48), CH_3COOH (after fragmentation in the ionization chamber: 44, 45, 46, 47); CS (44, 45, 46), BCl (45, 47), NO_2 (46, 48, 47), N_2O (44, 45, 46) (Bowen 1966; Charef and Sheppard 1984; De Groot 2011; Epstein et al. 1951; Epstein et al. 1953; Emiliani 1966; Mucciarone and Williams 1990; Weber et al. 1976). Among these, the absence of interference at m/z = 45 restricts the possible molecules to C_2H_5OH (46, 47, 48) and NO_2 (46, 48, 47). To characterize further this contamination, a few measurements of untreated calcite-yeast mixture samples (10%, 25%, 75%, and 90% of yeast) were performed using the classical method of McCrea (1950), which involves cryogenic purification of CO_2 from possible contaminants in a vacuum line. In our laboratory CO_2 purification is performed under vacuum at −130°C, and both C_2H_5OH and NO_2 are theoretically condensable. No significant shift (>0.2‰) was observed (Table 1), which indicates that the contaminant is cryogenically separated from CO_2 under vacuum at −130°C and that C_2H_5OH and/or NO_2 might be responsible for the interference at m/z 46. Their formal identification however remains to be performed.

For untreated dolomite–yeast mixtures, the results of the calculation using Eq. 1 are compared well with the measured data (Table 5 and Fig. 6). A negative shift is found for $\delta^{13}C_{CO2}$ in samples with a proportion of yeast ≥75% and none for $\delta^{18}O_{CO2}$ due to a smaller difference between yeast and dolomite $\delta^{18}O_{CO2}$. A contribution of CO_2 produced from the reaction of yeast with H_3PO_4 at 80°C is thus consistent with $\Delta^{18}O$ and $\Delta^{13}C$ values measured on dolomite–yeast mixtures and

there is no need for another interfering molecule. The fact that the interference observed on m/z 46 in calcite mixtures (H_3PO_4 digestion at 25°C) is not observed in dolomite mixtures (H_3PO_4 digestion at 80°C) suggests that the contaminating molecule is either not produced, or more likely, not stable at 80°C.

To summarize, for high proportion of immature organic matter relative to carbonate, two scenarios are possible depending on the temperature of H_3PO_4 digestion. At 25°C, CO_2 production from yeast reaction with H_3PO_4 is generally negligible. However, a contaminant species contributes to the signal on mass 46 (but not 45), impacting the measurement of $\delta^{18}O_{CO2}$ only. At 80°C, the amount of CO_2 generated by organic matter can impact both $\delta^{13}C_{CO2}$ and $\delta^{18}O_{CO2}$ measurements when the organic over carbonate mass ratio is high, provided that its $\delta^{13}C$ and $\delta^{18}O$ values are different from those of the carbonate sample.

Evaluating the need for organic matter removal

For C isotope analyses: $\delta^{13}C_{CO2}$ shifts result from the contribution of CO_2 produced by yeast during H_3PO_4 digestion. In the case of H_3PO_4 digestion at 25°C for 4 h, our results show that the production of CO_2 from yeast is too small to impact $\delta^{13}C_{CO2}$ analyses. Using Eq. 1 for a sample with 95% of yeast, a significant $\delta^{13}C_{CO2}$ shift ($\Delta^{13}C > 0.2‰$) can only be obtained if the difference between carbonate and organic matter $\delta^{13}C_{CO2}$ values reaches ~350‰, which never happens in natural systems. Therefore, organic matter removal of a specific sample is not required if carbonate is dissolved at 25°C for 4 h (for instance calcite, aragonite, vaterite, hydromagnesite, or magnesite). In the case of H_3PO_4 digestion at 80°C for 2 h, a larger amount of CO_2 is produced by yeast. A significant $\delta^{13}C_{CO2}$ shift is produced for dolomite–yeast samples with a proportion of yeast ≥75% (Table 2 and Fig. 2A). Our previous work demonstrated that at even higher temperature (i.e., 130°C), the proportion of CO_2 produced by H_3PO_4 digestion of yeast increases so that $\delta^{13}C_{CO2}$ shifts are observed for yeast proportions as low as 25% (Lebeau et al. 2014). Hence, organic matter from organic-rich natural samples must be removed more cautiously for C isotope analysis of carbonate when acid digestion is performed at high temperature.

For O isotope analyses: $\delta^{18}O_{CO2}$ shifts are only observed for calcite samples with a proportion of yeast higher than 25% (Table 1 and Fig. 1B). These isotopic shifts result from a gaseous contaminant (possibly C_2H_5OH and/or NO_2) produced by the reaction of organic matter with phosphoric acid at 25°C. Its effect on O isotope composition is poorly reproducible and starts at relatively low proportions of organic matter. No shift in $\delta^{18}O_{CO2}$ is observed for dolomite samples, although a contribution of CO_2 issued from the reaction of yeast with H_3PO_4 at 80°C is demonstrated from $\delta^{13}C_{CO2}$ results. The absence of shift likely results from a coincidence, $\delta^{18}O_{CO2}$ values of the CO_2 produced by yeast being similar to that produced by dolomite during H_3PO_4 reaction.

Our results on calcite and dolomite thus indicate that $\delta^{13}C_{CO2}$ and $\delta^{18}O_{CO2}$ may be shifted by the presence of organic matter but only when its proportions relative to carbonate are higher than 75% for $\delta^{13}C$ and 25% for $\delta^{18}O$. The finding that below 25% of organic matter no shift is observed is compatible with most previous studies (e.g., Allison et al. 1996; McConnaughey 1989; Nagtegaal et al. 2012; Oehlerich et al. 2013; Sarkar et al. 1990; Serrano et al. 2008; Watanabe et al. 2001; Zhang et al. 2001) in which no significant shifts was observed between treated and untreated samples. Few cases of $\delta^{13}C$ and/or $\delta^{18}O$ shifts have nonetheless been reported (e.g., Allison et al. 1996; Boiseau and Juillet-Leclerc 1997; Grottoli et al. 2005; Land et al. 1975; Wierzbowski 2007). In the light of our results, a possible explanation for these shifts may be that the treatments failed at efficiently removing organic matter and instead increased the reactivity of organic matter with H_3PO_4 (as shown here in H_2O_2 treatments; Figs. 1 and 2). In those cases, the shifts might thus be due to adverse effects of the treatment on the reactivity of organic matter during carbonate digestion.

To summarize, organic matter removal is useless when the proportion of organic matter relative to carbonate is equal to or lower than 25%, even for acid digestion at 80°C. Above 25%, isotopic shifts may occur except for calcite $\delta^{13}C_{CO2}$. The reactivity of immature organic matter during acid digestion at a given temperature is probably strongly variable from one sample to another. In the case of organic matter proportions relative to carbonate higher than 25%, it is recommended to test on a few samples if organic matter removal is necessary. These tests are usually performed by evaluating the impact of organic matter removal treatment on the measured $\delta^{18}O$ and $\delta^{13}C$ values, which is time consuming. Our results show that a strong deterioration of the reproducibility is correlated with the isotopic shifts for both $\delta^{13}C_{CO2}$ and $\delta^{18}O_{CO2}$ (Tables 1 and 2; Fig. 5). A poor reproducibility can thus be used as an indicator of the need for removing organic matter from the samples.

Comparison of treatments for organic matter removal

In the absence of yeast, none of the treatments tested in the present contribution altered the isotope composition of pure carbonates, as expected from previous works (Emiliani 1966; Epstein et al. 1951, 1953; Mook, 1971; Wierzbowski 2007). For mixture samples, the efficiencies of yeast removal and reduction of the isotopic shifts induced by its presence vary from one treatment to the other. H_2O_2 treatment is not adapted to samples with large proportion of organic matter. Indeed, yeast is not quantitatively removed when its proportion is higher than 50% (Figs. 3A and 4A). This result is consistent with those of previous studies, suggesting that H_2O_2 is less efficient than NaOCl (Falster et al. 2018; Gaffey et al. 1991; Gaffey and Bronnimann 1993; Wierzbowski 2007). Moreover, our study demonstrates that after treatment, yeast is more reactive to H_3PO_4 digestion. The proportion of CO_2 produced by pure yeast increased by more than 27 times after treatment both at 25°C and 80°C ($\mu_{H2O2} = 27 \times \mu_{untreated}$) (* in Tables 1 and 2). The carbonate C and O isotope values of mixture

samples were more strongly shifted after treatment. For instance, the $\Delta^{13}C$ of dolomite with 95% of yeast is only of −0.66‰ without treatment and increases sharply to −6.54‰ after H_2O_2 treatment (Table 2). The isotope mass balance in Eq. 1 allows us to calculate $\delta^{13}C_{CO2}$ and $\delta^{18}O_{CO2}$ of mixture samples treated by H_2O_2 based on the quantity and isotope composition of CO_2 released by pure yeast and pure carbonates. For dolomite samples, the results of this calculation compare well with the measured data since negative shifts in $\delta^{13}C_{CO2}$ and $\delta^{18}O_{CO2}$ are obtained for samples containing more than 75% of yeast (Table 5 and Fig. 6). The calculated shifts are larger than the measured values, probably reflecting inaccurate determination of the isotope compositions and μ of yeast treated by H_2O_2 (only one measurement). Nonetheless they are consistent with a contribution of CO_2 produced by yeast treated with H_2O_2. As for untreated samples, this explanation does not account for the behavior of calcite samples. Although at 25°C, the yeast treated by H_2O_2 is also more reactive than untreated yeast, the shifts measured in $\delta^{18}O_{CO2}$ are not predicted by the calculation from Eq. 1, suggesting the presence of others molecules (Table 4 and Fig. 6B). Finally another drawback of H_2O_2 treatment is the significant carbonate dissolution, as previously documented (Falster et al. 2018; Gaffey and Bronnimann 1993; Nagtegaal et al. 2012; Pingitore et al. 1993; Wierzbowski 2007). This is illustrated in this study by a higher mass loss after treatment than the initial mass proportion of yeast (Figs. 3B and 4B). This may be an issue for natural samples with isotopically heterogeneous carbonate grains. In that case, carbonate dissolution may be prevented by using an alkaline H_2O_2 solution (Fallet et al. 2009; Falster et al. 2018).

In the present contribution, the NaOCl treatment did not remove organic matter quantitatively (as also noted by Charef and Sheppard 1984) but was much more efficient than H_2O_2 and LTA (Figs. 3A and 4A). In the worst case, less than 25% of the initial amount of yeast remained after treatment and most importantly, $\delta^{13}C_{CO2}$ and $\delta^{18}O_{CO2}$ values of treated mixture samples were indistinguishable from those of untreated pure carbonates, i.e., calcite R0 and dolomite INYO. Although previous studies reported no appreciable dissolution of calcium carbonate by NaOCl treatment (Gaffey and Bronnimann 1993; Nagtegaal et al. 2012; Pingitore et al. 1993), our experimental results clearly demonstrate carbonate dissolution, yet to a smaller extent than H_2O_2 (Figs. 3B and 4B). It is thus not recommended to use NaOCl treatment for small and/or isotopically heterogeneous samples, unless the pH of the solution can be made more alkaline (e.g., Nagtegaal et al. 2012).

The low-temperature oxygen plasma treatment removes less organic matter than NaOCl but more than H_2O_2 (Figs. 3A and 4A). The yeast reactivity to H_3PO_4 digestion is increased by LTA although less than by H_2O_2, with a production of CO_2 five times more important than untreated yeast ($\mu_{plasma} = 5 \times \mu_{untreated}$) (* in Table 2). However, organic matter removal by LTA is efficient enough so that $\delta^{13}C_{CO2}$ and $\delta^{18}O_{CO2}$ of the mixture samples are indistinguishable from those of untreated pure carbonates R0 and INYO. This is also supported by the good agreement in isotope mass balance between predicted and measured CO_2 in yeast–dolomite mixtures (Table 5 and Fig. 6). A significant exception is observed for dolomite samples with 95% of yeast that present a $\Delta^{18}O$ of −0.45‰ after 8 h of LTA treatment, and −0.21‰ after 15 h of treatment (Table 5). The main advantage of plasma ashing is that it occurs in dry conditions, hence preventing carbonate dissolution and allowing perfect preservation of carbonates: only 1% of mass loss occurs for pure carbonate samples after LTA treatment while wet treatments induce between 6% and 40% of mass loss (Tables 1 and 2; Figs. 3 and 4). This method thus offers a possibility to remove organic matter from carbonate-poor samples while allowing the quantification of the carbonate content in the initial sample, provided that the mass loss induced by LTA treatment is quantified.

Conclusion

In the present work, we compared the three classical treatment used for organic matter removal. Our aim was to evaluate both their necessity and usefulness for calcite or dolomite isotope measurements using the CF-IRMS method, which has nearly completely replaced dual inlet IRMS methods in stable isotope laboratories worldwide. The need for organic matter removal in carbonate samples was evaluated for a wide range of immature organic matter proportions, with a focus on the extremely high proportions under-investigated so far.

Our results show that when using CF-IRMS, no treatment is necessary for organic matter over carbonate ratios lower than 50%, i.e., for most natural samples. For higher ratios, we recommend to test if organic matter removal is necessary especially when a poor reproducibility is observed on $\delta^{13}C_{CO2}$ and $\delta^{18}O_{CO2}$ measurements of untreated samples. In the present experiments that use yeast as a model for immature organic matter, the results show that for untreated calcite mixtures, the $\delta^{13}C_{CO2}$ values were never affected while $\delta^{18}O_{CO2}$ can be shifted (up to +2‰) for yeast proportions higher than 50%. For dolomite, $\delta^{13}C_{CO2}$ values were slightly shifted (down to −0.6‰) only for yeast proportions higher than 75%. $\delta^{18}O_{CO2}$ values were not affected because of a similarity in the $\delta^{18}O_{CO2}$ values of dolomite and yeast but this may not always be the case.

Our comparison of the three different treatments shows that for samples extremely rich in organic matter, NaOCl is the most efficient procedure, while H_2O_2 is the least efficient. In terms of carbonate preservation, H_2O_2 showed a strong dissolution of carbonates, NaOCl a mild dissolution, while LTA preserved them perfectly. Finally, both NaOCl and LTA treatments provide precise and accurate isotope results on carbonates while H_2O_2 treatments strongly increased the reactivity of the remaining organic matter toward H_3PO_4, therefore strongly worsening both C and O isotopic shifts.

Our results also provide constraints on the mechanisms responsible for isotope offsets linked to high proportions of organic matter relative to carbonates. During acid digestion at 25°C, yeast generated a negligible amount of CO_2 and more

importantly a volatile contaminant. This contaminant was not efficiently separated from CO_2 by chromatography and interfered on the mass 46 (m/z) shifting only $\delta^{18}O_{CO2}$ values, but was separated by cryogenic purification at −130°C under vacuum. Future work should focus on identifying this contaminant and improving its chromatographic separation from CO_2. The use of an appropriate column should be considered once it has been identified. For example, a column Molesieve 5A can separate CO_2 from NO_2. Finally, when carbonates are digested at 80°C, immature organic matter does not release this contaminant but generate much more CO_2 than at 25°C, which is added to the CO_2 produced by carbonate dissolution. This may be a particular concern for methods based on calcite digestion at temperature of 70°C or higher.

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