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Immobilization of microalgae cells in alginate facilitates isolation of DNA and RNA☆

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ABSTRACT

Isolation of nucleic acids from *Chlorella* is difficult, given the chemically complex nature of their cell walls and variable production of metabolites. Immobilization of microalgae in polymers adds additional difficulty. Here, we modified, amended, and standardized methods for isolation of nucleic acids and compared the yield of DNA and RNA from free-living and encapsulated microalgae *C. sorokiniana*. Isolation of nucleic acids from immobilized cells required two steps in dissolving the alginate matrix, releasing the cells, and mechanical disruption with glass beads. For DNA extraction, we used modified versions of a commercial kit along with the hexadecyltrimethylammonium bromide (CTAB) method. For RNA extraction, we used the commercial TRI reagent procedure and the CTAB-dithiotreitol method. Quantity and quality of nucleic acids in extracts varied with growth conditions, isolation procedures, and time of incubation of the original culture. There were consistently higher amounts of DNA and RNA in extracts from immobilized cells. Quantitatively, the modified procedure with the commercial Promega kit was the most reliable procedure for isolating DNA and a modified commercial TRI reagent procedure was the choice for isolating RNA. All four procedures eliminated proteins efficiently and had low levels of contamination from residual polysaccharides from the matrices and/or metabolites naturally produced by the microalgae. All DNA extracts under both growth conditions, time of incubation, and two isolation methods successfully amplified the 18S ribosomal RNA by PCR and quantitative reverse transcription (RT-qPCR).

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1. Introduction

Encapsulating microbial cells in polymers is a common technique in fundamental research and industrial applications. These include production of probiotics (Anal and Singh, 2007), inoculants to increase crop production (Bashan and de Bashan, 2016; Bashan et al., 2014; Schoebitz et al., 2013), potential applications in removing pesticides from soils (Fuentes et al., 2013), and remediating sewage and industrial effluents (Cohen, 2001; Cruz et al., 2013; Gadd, 2009). Immobilization of algal cells in polymers is a feasible technology that efficiently removes heavy metals and nutrients from contaminated water (de Bashan and Bashan, 2010; de Bashan et al., 2004; Lebeau and Robert, 2006; Moreno-Garrido, 2008; Yabur et al., 2007). Additionally, immobilization is used as a tool to create and study synthetic mutualism between microalgae and bacteria (de Bashan and Bashan, 2008; de Bashan et al., 2008a, 2008b, 2011, 2015, 2016; Meza et al., 2015a, 2015b; Palacios et al., 2016a, 2016b, 2017).

Chlorella spp. are one of the most studied microalgae for fundamental studies (Krienitz et al., 2015) and biotechnological applications (Perez-Garcia and Bashan, 2015). Most of the fundamental studies analyze physiological changes by metabolite production of immobilized cells compared to planktonic cells (de Bashan et al., 2015); however, new approaches investigating changes at the gene-level involve the extraction of DNA and RNA from immobilized cells. In spite of the increasing biotechnological applications of encapsulated microalgae, to the best of our knowledge, there are no procedures for isolating nucleic acids that are useful for biological evaluation of microalgae cells immobilized in polysaccharide-based matrices. Methods for isolation and purification of nucleic acids fall into two categories: (a) the solution-based methods and, (b) the column-based protocols (Tan and Yiap, 2009), with the later increasingly applied for microalgae, however, it requires considerable amounts of algal biomass. In general, isolation of nucleic acids from algae has inherent technical difficulties resulting from their structurally robust and chemically complex cell walls (Gerken et al., 2013). Frequently, procedures to isolate nucleic material from some algal groups fail (Varela-Álvarez et al., 2006). For example,

☆ Dedication: this study is dedicated to the memory of Dr. Michael Schmid (Big Mike) (1968-2016) of Helmholz Zentrum Münich, Neuherberg, Germany

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coccoid microalgae, especially *Chlorella* spp., have rigid cell walls that make them difficult to disrupt (Fawley and Fawley, 2004). Thus, an additional initial stage of cell disruption in most protocols has to be adapted for each algal species. These steps include mechanical homogenization, enzymatic digestion, a combination of these methods (Gerken et al., 2013), and sometimes these steps are followed by boiling to facilitate cell lysis (Tear et al., 2013). In addition, polysaccharides, lipids, and phenolic compounds typically produced by microalgae in abundance interfere with isolating high quality nucleic acids and with down-stream enzymatic reactions (Thanh et al., 2009; Varela-Álvarez et al., 2006). To solve these isolation problems, solution-based protocols use acetyltrimethylammonium bromide (CTAB) that efficiently separates polysaccharides, while soluble proteins and other material are separated by solvents and centrifugation (Sambrook and Russell, 2001). Alternatively, guanidinium thiocyanate-phenol-chloroform extraction reagent is used to remove proteins, lipids, and carbohydrates from RNA of *Chlorella* (Qian et al., 2008).

Our hypothesis was that immobilizing microalgae in alginate interferes with procedures to extract nuclear material from microalgae. Our objectives were to: (a) standardize isolation methods for nucleic acids, (b) determine which extraction methods were more consistent for their capacity to extract nucleic acids from encapsulated cells, and (c) measure the level of interference of the immobilization procedure in the nucleic acid extraction procedures.

2. Materials and methods

2.1. Microorganisms and cultivation methods

This study used free-living planktonic (FC) and immobilized cells (IC) of the unicellular microalga *Chlorella sorokiniana* Shih. et Krauss (UTEX 2714, University of Texas, Austin, TX, formerly *C. vulgaris* UTEX 2714; Bashan et al., 2016). The inoculum was cultivated in sterilized C30 mineral medium ($g \cdot L^{-1}$): KNO_3 (25); $MgSO_4 \cdot 7H_2O$ (10); KH_2PO_4 (4); K_2HPO_4 (1); $FeSO_4 \cdot 7H_2O$ (1); micronutrients ($\mu g \cdot L^{-1}$) H_3BO_3 (2.86); $MnCl_2 \cdot 4H_2O$ (1.81); $ZnSO_4 \cdot 7H_2O$ (0.11); $CuSO_4 \cdot 5H_2O$ (0.09); $NaMoO_4$ (0.021) for 5 days under continuous agitation (120 rpm), light intensity of 60 $\mu mol \cdot m^{-2} s^{-1}$, at 27 ± 1 °C (de Bashan et al., 2004). For the experiments, five-day-old cultures were harvested by centrifugation at 10,000 ×*g* for 5 min and washed three times in sterile 0.85% saline solution. In the treatment of free cells, 100 mL algal suspension in saline solution were directly inoculated in 200 mL synthetic growth medium (SGM) containing (in $mg \cdot L^{-1}$): NaCl (7), $CaCl_2$ (4), $MgSO_4 \cdot 7H_2O$ (2), K_2HPO_4 (217), KH_2PO4 (8.5), Na_2HPO_4 (33.4), NH_4Cl (191) (de Bashan et al., 2011) and then divided into three flasks for incubation in the same medium. For the treatment of immobilized cells, algal cells were immobilized, using the procedure described in de Bashan et al. (2004). Briefly, 20 mL of algal suspension containing 6.0×10^6 cells mL^{-1} were mixed by stirring with 80 mL sterile 1.4% (*w*/*v*) suspension of sodium alginate (154,723 MP Biomedicals, Santa Ana, CA) for 15 min. The microalgae-alginate suspension was dripped into 2% $CaCl_2$ solution under slow stirring. Beads (3–4 mm diameter) were formed, using automated bead-forming equipment (de Bashan and Bashan, 2010). The beads remained for 1 h at 22 ± 2 °C for curing, then washed with sterile 0.85% saline solution (Gonzalez and Bashan, 2000), and transferred to 250 mL Erlenmeyer flasks containing 100 mL SGM. The flasks containing immobilized cells or free cells were incubated in SGM for 48 h or 72 h under the same conditions for culturing microalgae described earlier.

2.2. Preparation of samples for nucleic acid isolation

To release the cells before isolating nucleic acids, 10 mL alginate beads containing microalgae were dissolved in citrate buffer pH 8.0 (sodium citrate 55 mM, 30 mM EDTA anhydrous, and 150 mM sodium chloride) at 1:4 bead/buffer, v/v under circular agitation (150 rpm, ~15 min). The alginate–microalgae suspension was centrifuged at 10,000 ×*g* for 5 min. The pellet was washed three times in the same buffer and was re-suspended in 1 mL 0.85% saline solution. Free cells were directly centrifuged, washed three times in citrate buffer and re-suspended to a final volume of 1 mL 0.85% saline solution per each 5 mL of the original culture in SGM. Algal suspensions from both treatments were stored at −80 °C up to one week before processing. Freezing at this temperature also caused weakening of the cell walls after thawing the samples, in preparation for nucleic acid isolation. Handling of samples for RNA isolation was performed at 4 °C. The number of cells was obtained individually for each isolated sample, considering variations of algal density between free cells and immobilized cells and the increase of population after incubation. Cells were counted in a Neubauer hemocytometer under a light microscope connected to an image analyzer (Image ProPlus 6.3; Media Cybernetics, Rockville, MD). Ten fields were counted to calculate the number of cells per isolation sample (1 mL) (Gonzalez and Bashan, 2000).

2.3. DNA extraction from free and immobilized cells

DNA was isolated after 48 and 72 h of incubation with SGM. All the procedures tested for nucleic acid isolation were modifications of the original procedures. For DNA isolation, the main modification was aggressive mechanical disruption of cells with vortexing and heat, using sterile glass beads (425–600 μm, G8772, Sigma-Aldrich).

Genomic DNA was extracted by two methods, Promega and Fawley-CTAB. The first method is a modification of the Wizard Genomic DNA purification Kit (A1120, Promega, Madison, WI) and the latter is a modification of the CTAB method for microalgae by Fawley et al. (1999).

2.3.1. Promega procedure

Lysis of cells was performed, as follows: 0.5 g sterile glass beads and 500 μL nuclei lysis solution (A7941, Promega) were added to 400 μL of the algal suspension and vortexed for 10 min at maximum speed. The homogenate was incubated at 80 °C for 5 min and vortexed for 5 min. The rest of the procedure, including RNase treatment, protein precipitation, DNA precipitation, and elution were performed according to kit instructions. Briefly, proteins were precipitated for 15 min at 4 ± 1 °C, followed by centrifugation at 11,000 *g* for 3 min. DNA was precipitated in ice-cold isopropanol at 16,000 *g* for 5 min and washed with 70% ice-cold ethanol.

2.3.2. Fawley-CTAB procedure

The algal suspension was washed once with 100 μL extraction buffer (1 M NaCl, 70 mM TRIS HCl, 30 mM Na_2EDTA, pH 8.1) and resuspended in 100 μL of the same buffer. Cell disruption was performed with 0.5 g glass beads added to the algal suspension and vortexed for 1 min at full speed. Then, 12 μL 10% (CTAB; hexadecyltrimethylammonium bromide; 6269, Sigma-Aldrich, St. Louis, MO) was added and the sample heated in boiling water for 1 min. After the lysate cooled, 2 μL RNase (A797C, Promega) (4 $mg \cdot mL^{-1}$) were added and incubated at 37 °C for 10 min. Further steps were performed, mostly as described by the reference method of Fawley et al. (1999). Briefly, after heating the sample, 200 μL 100% chloroform were added, gently mixed by inverting the tube for 10s and centrifuged at 2000 ×*g* for 2 min. About 100 μL were recovered from the aqueous phase, mixed with 170 μL 0.5% CTAB in 40 mM NaCl, and centrifuged at 12,000 ×*g* for 5 min. The pellet containing the DNA was re-suspended in 100 μL 1.2 M NaCl, precipitated with 250 μL 100% ice-cold ethanol, and kept at −20 °C for 90 min. Then, the sample was centrifuged at 16,000 ×*g* for 10 min and eluted with DNA rehydration solution (A796A, Promega).

2.4. RNA extraction from free and immobilized cells

RNA was isolated from cells harvested after 72 h incubation. Before RNA isolation, the samples were prepared by dissolving the alginate

beads and/or washing the algal suspensions, as described earlier. Two procedures for RNA isolation were tested: (1) The common protocol of phenol-chloroform, using the TRI Reagent (93,289, Sigma-Aldrich) and (2) CTAB–DTT, an alternative method proposed by Thanh et al. (2009) that combines several procedures for isolating RNA from green algae, using CTAB- DTT (dithiothreitol) chlroroform:isoamyl alcohol, 2-mercaptoethanol, and LiCl, as the most important reagents. Optimization of both methods involved slight modifications, essentially in the steps of cell disruption, as will be described later. All materials used were either sterilized by autoclave or wiped with RNaseZAP (R2020, Sigma-Aldrich) and diethylpyrocarbonate (DEPC-treated water) (D5758, Sigma-Aldrich) to remove RNAse.

2.4.1. Modified RNA isolation with TRI reagent

The microalgae suspension was mixed with 0.5 g glass beads (425–600 μm), frozen in liquid nitrogen, and ground by mortar and pestle. Immediately, 600 μL TRI Reagent were added and vigorously vortexed at top speed for 25 s without allowing the sample to completely melt. The homogenate was centrifuged at 11,000 ×g for 15 min. About 500 μL of the upper aqueous phase were recovered and placed in a new microtube. An ice cold volume of 125 μL 100% chloroform (C2432, Sigma-Aldrich) was added and mixed gently by inversion and incubated for 3 min at room temperature (23 °C). The sample was centrifuged at 12,000 ×g for 15 min. The upper aqueous phase, containing the RNA, was placed in a new microtube containing ice cold 250 μL 100% isopropanol, mixed gently by inversion, and incubated for 10 min. The sample was centrifuged at 13,000 ×g for 15 min and the supernatant was discarded. The pellet was washed with 100 μL ice cold 75% ethanol in DEPC-treated water and centrifuged at 14,000 ×g for 8 min. The supernatant was discarded and the remaining ethanol was evaporated under sterile conditions in a laminar flow hood. The RNA was eluted in DEPC-treated water and incubated for 5 min at 65 °C. Unless indicated, all steps, including centrifugation, were performed at 4 °C.

2.4.2. Modified Thanh protocol (CTAB–DTT)

Glass beads (0.05 g; 425–600 μm, Sigma-Aldrich) were added to the microalgae suspension. The suspension was frozen in liquid nitrogen and pulverized with mortar and pestle. Then 500 μL of the extraction buffer (100 mM Tris-HCl at pH 8.0; 1.4 M NaCl; 20 mM EDTA at pH 8.0; 2% w/v CTAB; and 50 mM DTT [646,563, Sigma-Aldrich]) were added. The homogenate was vigorously mixed by vortexing at high speed for 10s, without completely melting the sample. An aliquot of 500 μL 100% chloroform-isoamyl alcohol 24:1 v/v (C0549, Sigma-Aldrich) was added to the homogenate and mixed thoroughly by inversion. The homogenate was centrifuged at 12,000 ×g for 15 min at 20 °C. About 500 μL of the upper aqueous phase was transferred to a new tube and 150 μL (0.3 vol) of ice cold 100% ethanol were added and gently mixed by inversion. This mixture was incubated for 2 min and transferred to a new tube containing 162 μL (0.25 vol.) of 12 M LiCl that was previously mixed with 2-mercaptoethanol (63,689, Sigma-Aldrich) to a final concentration of 1% in LiCl. The sample was mixed by inversion. RNA underwent precipitation for 1 h at −80 °C, followed by centrifugation at 15,000 ×g for 30 min. The supernatant was discarded and the pellet was eluted with 100 μL DEPC-water and 25 μL (0.25 vol.) of 12 M LiCl were added and mixed by pipetting. RNA was re-precipitated for 1 h at 4 °C and centrifuged, as in the previous step. The pellet was washed with 200 μL 75% ethanol in DEPC-treated water and centrifuged at 16,000 ×g for 10 min. The RNA was eluted in 50 μL DEPC-treated water and incubated at 65 °C for 5 min and 330 rpm in a thermal mixer (Eppendorf, Hamburg, Germany). Unless indicated, all steps, including centrifugation, were performed at 4 °C.

2.5. Determination of yield of nucleic acid

Quantification of nucleic acid was done in a spectrophotometer (NanoDrop 2000, Thermo Scientific, Waltham, MA). For each sample, the yield of nucleic acids per cell was calculated, as follows: Nucleic acid yield (ng × cell) = [(ng of total nucleic acid × sample) / (number of cells × sample)].

2.6. Assessment of quality of nucleic acids

Integrity of RNA and DNA was determined by performing electrophoresis in 1.5% agarose TBE gels at 100 V for 1 h and stained (SYBR Gold, Invitrogen, Carlsbad, CA). The purity of DNA and RNA was determined by the 260/280 and 260/230 absorbance ratio, based on the maximum absorbance of nucleic acids at 260 nm. Therefore, a decrease in the 260/280 and 260/230 nm ratios was used as an indicator of increased amounts of impurities (Sambrook and Russell, 2001). In addition, representative spectrophotometric scanning profiles were selected to illustrate the quality of nucleic acid extracts.

2.7. PCR of 18S rDNA

To determine the possible impact of carryover inhibitors during PCR, the extract of DNA was tested for PCR amplification of the 18 s rDNA, using the primers reported by Wan et al. (2011), 18S F (5′-CCTGCGGCTTAATTTGACTC-3′) and 18S R (5′-GCGAACCAACCGTGACTATT-3′). Each PCR reaction contained 2.5 μL 10× reaction buffer (203,205, Qiagen), 1.25 μL DMSO (D8418, Sigma-Aldrich), 2.5 μL bovine serum albumin at 1 $\mu g \cdot \mu L^{-1}$ (R3961, Promega), 0.5 μL 200 mM dNTP mix (D7295, Sigma-Aldrich), 1 μL of each primer at 5 μM per primer, 0.125 μL HotStar DNA polymerase at 5 $U \cdot \mu L^{-1}$ (203,205, Qiagen), 1 ng DNA template (2 μL), and 14.1 μL sterile ddH_2O. Amplification was carried out in a thermal cycler (Life Technologies, Carlsbad, CA), by preliminary denaturation of DNA at 95 °C for 10 min, followed by 35 cycles of DNA template denaturation at 95 °C for 30 s, primer annealing at 60 °C for 45 s, initial extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min.

2.8. Two step RT-qPCR

RNA extracts were tested for RT-qPCR amplification of 18S rRNA. RNA was treated with deoxyribonuclease DNase I (AMPD1, Sigma-Aldrich) and then used for cDNA synthesis, using a reverse transcription system (A3500, Promega). A reference standard curve for the 18S rRNA was prepared, using fresh culture of microalgae and the RNA extracted by the TRI Reagent procedure. Serial dilutions 1:10 of the reference cDNA and primer concentration were optimized to maximum efficiency (97% efficiency, $r^2 = 0.99$, 5 dilutions, $n = 3$).

For the qPCR experiment, 10 μL volume reactions contained 5 μL 2 × SSo advanced SYBR Green supermix (Bio-Rad Laboratories, Hercules, CA), 0.1 μL each of forward and reverse primers, with 5 μM and 3.8 μL water and 1 μL cDNA equalized at 233 $ng \cdot \mu L^{-1}$. This corresponds to the dynamic range established by the reference standard curve. Three qPCR reactions were performed for each treatment of growth condition (free cells and immobilized cells) and isolation procedures and the experiment was repeated three times. Amplification was done in the StepOne Real-Time PCR System (4,376,600, Applied Biosystems, Carlsbad, CA) under the following thermal conditions: polymerase activation at 95 °C for 30 s, 40 cycles at 95 °C for 15 s each, 60 °C for 45 s, and final extension at 95 °C for 15 s. Negative controls, consisting of RNA that was free of DNA, were included in each PCR run. The specificity of amplification was confirmed by melting-curve analysis from 60 °C to 95 °C and the final products were visualized in 1.7% agarose TBE gel stained with SYBR Gold (S11494, Thermo Scientific).

2.9. Experimental design and statistical analysis

Batch cultures were used for all experiments. Nucleic acids were extracted from microalgae cultured under two growth conditions: (1)

free-living cells and (2) immobilized cells. Each condition consisted of three 250 mL Erlenmeyer flasks containing 100 mL SGM, as experimental replicates. Samples for isolation procedures were taken from each Erlenmeyer flask, triplicate samples for DNA and four samples for RNA. In treating free cells, each sample was derived from 5 mL of algae suspension. In treating immobilized cells, 10 g of alginate beads was used. DNA was isolated after 48 and 72 h of incubation and RNA after 72 h. Each experiment was repeated twice to demonstrate consistency of results.

All data were checked for normality by the Shapiro-Wilk test and then by paired comparisons, using two-tailed Student's *t*-test, which were performed between each pair of cell conditions within each isolation procedure and time of incubation. Comparisons for DNA/RNA were based on the calculated value of yield (ng) of nucleic acids per million cells. Data from RT-qPCR were analyzed by comparison of cycle threshold (Ct) values, following the approach of Lykidis et al. (2007).

3. Results

The general conditions for all comparisons were: nucleic acids were isolated from *C. sorokiniana* grown as free cells (FC) or immobilized in alginate beads (IC). Two modified extraction methods were used to obtain DNA (Promega and Fawley-CTAB) and RNA (TRI and CTAB–DTT).

3.1. DNA

DNA was successfully isolated from *C. sorokiniana* by two procedures at two incubation times under two growth conditions (Figs. 1, 2). The quantity of DNA was not statistically tested across incubation time; however it decreased with time, with higher yields (ng per million cells) under incubation for 48 h. This was more evident using the Promega kit than using the Fawley-CTAB method.

The Student's *t*-test regarding the quantity of extracted DNA revealed consistent differences between cell conditions at each incubation time, regardless of the isolation method (Fig. 1). Using the Promega kit, at 48 h, immobilized cells yielded significantly more DNA (177.95 ± 15 ng $\times 10^6$ cells) than free cells (72.20 ± 12 ng $\times 10^6$ cells), $p < 0.0001$ and similar results were obtained at 72 h of incubation ($p = 0.024$) but with considerable lower yields (IC $= 19.80 \pm 5$ ng $\times 10^6$ cells, FC $= 7.84 \pm 0.8$ ng $\times 10^6$ cells, Fig. 1A). Consistently, the Fawley-CTAB procedure recovered more DNA from immobilized cells than free cells at both incubation times. At 48 h, IC $= 131.85 \pm 9$ ng $\times 10^6$ cells and FC $= 95.45 \pm 12$ ng $\times 10^6$ cells ($p < 0.03$) and similarly, at 72 h, IC $= 103.71 \pm 6$ ng $\times 10^6$ and FC $= 22.34 \pm 2$ ng $\times 10^6$ cells ($p < 0.0001$ (Fig. 1B).

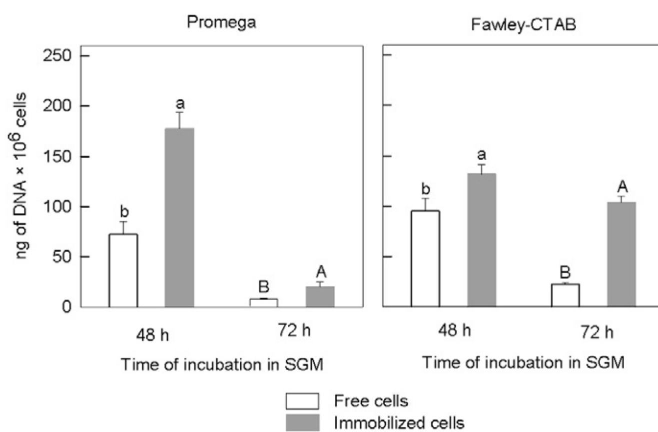

Fig. 1. DNA obtained from *Chlorella sorokiniana* UTEX 2714 grown under two growth conditions, at two incubation times, and two isolation procedures. Cell conditions: free cells or immobilized cells in alginate beads. DNA isolation procedures: (A) Promega = DNA isolation kit adapted for microalgae or (B) Fawley-CTAB = DNA isolation procedure modified from Fawley et al. (1999). Pair of columns denoted by different letters indicates significant difference at $P < 0.05$ by the two-tailed *t*-test. Bars indicate SE. $n = 9$.

3.1.1. Analysis of quality of DNA

Agarose electrophoresis confirmed genomic high molecular DNA (above 20 kb) under both growth conditions and the two isolation methods (Fig. 2). After 48 h, most samples had a well-defined band of DNA (Fig. 2A, B) and similarly after 72 h (Fig. 2B). At 72 h, the samples had more sheared DNA, as seen by a slight smear on the band. The purity of the DNA extract determined by the 260/280 and 260/230 absorbance ratios, showed that in general, the two assay procedures were efficient in removing interfering compounds, such as proteins. The average values of 260/280 ratios fluctuated around 2 in all samples, either obtained from FC or IC. In contrast, the 260/230 absorbance ratio had low values that indicated other contaminants were extracted with the DNA (Table 1). Even at lower values, the general trend indicated that the Fawley-CTAB procedure performed better at both incubation times. Representative absorbance curves for both growth condition illustrated these results (Fig. 2D, E). By either method of isolation, extracts of DNA from free cells showed the characteristic peak for nucleic acids at 260 nm; however, the presence of contaminants was revealed by an increase in the absorbance around 220 nm (Fig. 2D). Similar results were observed in extracts from immobilized cells, however, only the Fawley-CTAB procedure showed a more defined peak at 260 nm (Fig. 2E).

3.1.2. PCR

Gel electrophoresis of total DNA and 18S rDNA PCR products from *C. sorokiniana* under the different conditions are shown in Fig. 2C. Quantity and quality of the PCR product was similar between growth condition, isolations procedures, or length of incubation. Mean concentration for PCR products ranged from 453.56 ± 19.51 to 463.66 ± 27.62 ng·μL^{-1}. The quality of the PCR product measured by the 260/280 absorbance ratio (1.79 ± 0.01–1.80 ± 0.01) also reflected the quality of the original DNA template and thus maintaining lower 260/230 absorbance ratios (0.70 ± 0.04 to 0.71 ± 0.06).

3.2. RNA

Yield of RNA was consistently higher in the extracts from immobilized cells than from free cells ($p < 0.0001$) in both assayed methods (Fig. 3). When performing the TRI method the RNA extracted from immobilized cells was 393.11 ± 41 ng $\times 10^6$ cells, while free cells yielded 284.33 ± 18 ng $\times 10^6$ cells. The same trend but with considerable lower amounts was obtained by the CTAB-DTT method in free cells obtaining 108.1 ± 7 ng $\times 10^6$ cells and almost half of that amount in immobilized cells (46.5 ± 2 ng $\times 10^6$ cells).

3.2.1. RNA quality

Extracts from both extraction procedures exhibited strong bands, mainly showing the rRNAs. More bands were visualized in samples from immobilized cells compared to the free cells and consistently more bands in extracts obtained by the TRI Reagent method compared to CTAB-DTT method which also recovered some DNA (Fig. 4A, B). Nanodrop absorbance curves had similar patterns between both growth conditions and both isolation procedures and revealed strong contamination in the absorbance at 220–240 nm (Fig. 4C, D).

The overall pattern based on the 260/280 and 260/230 absorbance ratios indicated that RNA quality was similar in both growth conditions but noticeably different between isolation procedures. The quality of the extracted RNA in both growth conditions yielded 260/280 ratios ~2 indicating good quality of nucleic acids. When evaluating the 260/230 ratio at both growth conditions, the quality was lower than the expected (ratio ~ 2), and ranged between 0.2 and 0.9 with the higher values in the extraction by the CTAB-DTT technique (Table 2).

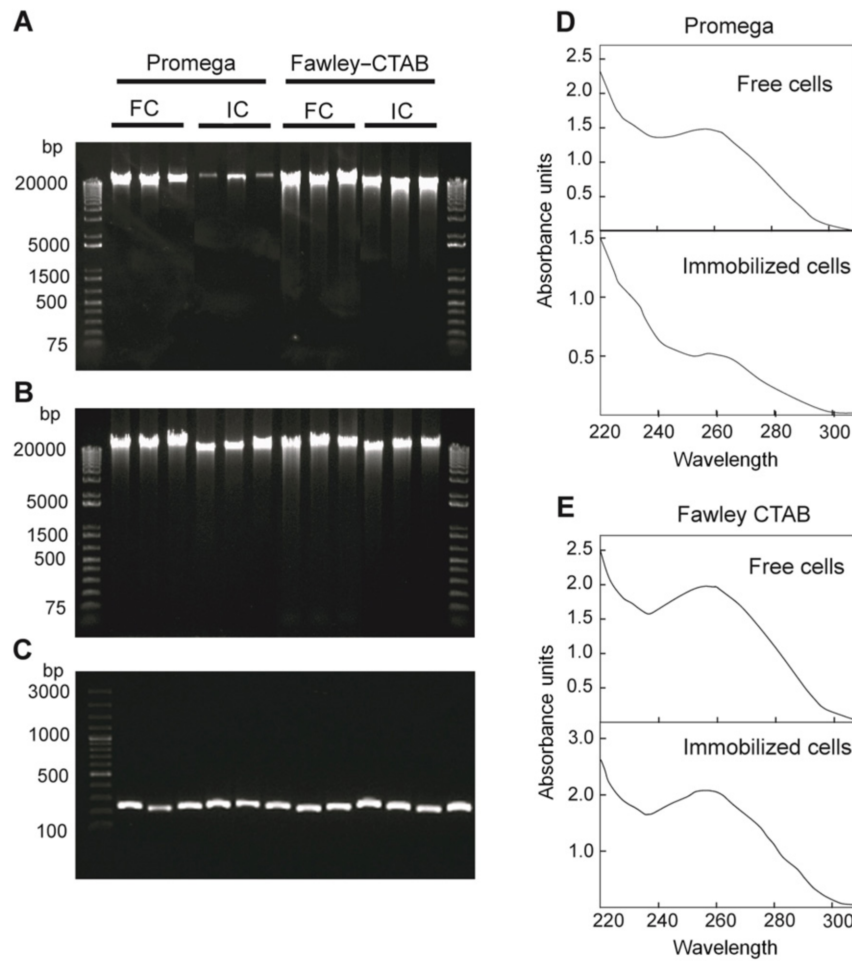

Fig. 2. Quality of genomic DNA and 18S rDNA from *Chlorella sorokiniana* UTEX 2714 grown as (FC) free cells or (IC) immobilized cells in alginate beads and two isolation procedures; Promega = DNA isolation kit adapted for microalgae or Fawley-CTAB = DNA isolation procedure modified from Fawley et al. (1999). Agarose gels for DNA extracts obtained after 48 h (A) or 72 h of incubation (B) and 18S DNA amplicons (C). Representative nanodrop absorbance curves for DNA extracts obtained by each isolation method (D, E). Spectrophotometric scan profiles acquired from Nanodrop. Each profile is representative of nine at each growth condition. Agarose gels were stained with SYBR gold.

3.2.2. RT-qPCR for the 18S gene

All cDNA templates produced positive amplification of the 18S rRNA gene. Among the samples, the cycle threshold (Ct) values ranged from 30.54 to 35.01, near the limit of detection (Fig. 5A). Significant differences were found between average the Ct of growth conditions ($p \leq 0.002$) showing variable PCR efficiencies depending on the isolation method assayed. For the TRI Reagent method the templates from free living cells amplified earlier (Ct = 30.67 ± 0.17) than templates from the immobilized cells (Ct = 34.39 ± 0.24), thus indicating higher PCR efficiencies in the former. The opposite happened with templates from CTAB-DTT method, where earlier Cts were detected in immobilized cells (Ct = 31.96 ± 0.24), compared to free living cells (Ct = 33.27 ± 0.17). Melting curves and gel electrophoresis of final PCR products confirmed one product of the expected size and no amplification in the negative controls (Fig. 5B).

4. Discussion

In spite of extraordinary advances in molecular procedures, we are still experiencing significant difficulties in obtaining high quality nucleic acids from green microalgae, especially from encapsulated cells. Some studies have described techniques to isolate DNA or RNA from free-living *Chlorella* spp. under common cultivation conditions (Fawley and Fawley, 2004; Wan et al., 2011); however, these techniques have shown inconsistencies when performed on encapsulated cells. The major challenges in nucleic acid isolation from free-living cells (planktonic) of *Chlorella* species are: (1) rigidity of the cell wall, (2) complex chemical composition of the cell wall that is rich in polysaccharides and glycoproteins, and (3) abundance of some metabolites that varies dramatically under different growing conditions (Gerken et al., 2013). All interfere with isolation procedures to different levels of magnitude.

Table 1
Absorbance ratios of the DNA extracted from *Chlorella sorokiniana* UTEX 2714 under two cell growth conditions and two isolation procedures.

Cell condition	260/280 ratio		260/230 ratio	
	Promega	F-CTAB	Promega	F-CTAB
Free cells	2.12 ± 0.1	1.97 ± 0.1	0.62 ± 0.3	0.73 ± 0.1
Immobilized cells	2.01 ± 0.1	2.46 ± 0.2	0.5 ± 0.1	0.93 ± 0.2

Average values ± SE.
$n = 9$.
Fawley-CTAB: DNA isolation procedure modified from Fawley et al. (1999).
Promega: DNA isolation kit adapted for microalgae.

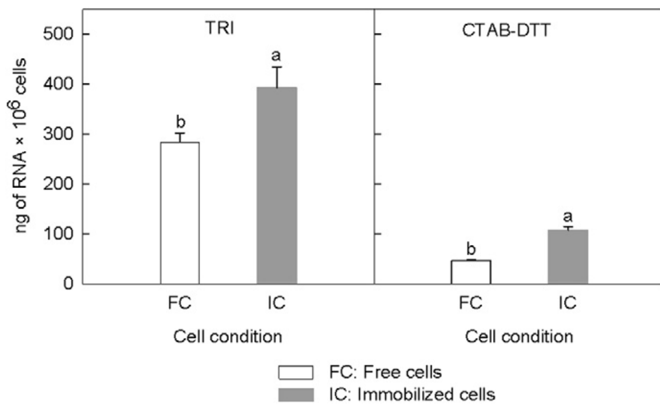

Fig. 3. RNA obtained from *Chlorella sorokiniana* UTEX 2714 grown under two growth conditions and isolated by two procedures. Cell conditions: FC = free cells or IC = immobilized cells in alginate beads; RNA isolation procedures: (A) TRI = RNA isolation procedure using TRI Reagent commercial product, (B) CTAB-DTT = RNA isolation procedure modified from Thanh et al. (2009). Columns denoted by different letters are statistically different at $P < 0.05$ by the two-tailed *t*-test. Bars indicate SE. $n = 12$.

Since encapsulation of microalgae is an evolving and promising technology for several biotechnological applications (de Bashan and Bashan, 2010; Perez-Garcia and Bashan, 2015), this study analyzed the effect of using immobilization with alginate on the quantity and quality of nucleic acids extracted from *Chlorella sorokiniana* UTEX 2714.

Our initial attempts to isolate genetic material involved direct extraction, using the commercial Promega kit or TRI Reagent procedures on cells immobilized in alginate beads. These extractions frequently failed to produce reliable and consistent yields of nucleic acids, compared with extractions from free-living cells. Yields were low, compared with extracts from other single cells, such as bacteria. The low yield from planktonic cells can be partly explained by the natural rigidity and plasticity of cell wall of *Chlorella* (Gerken et al., 2013). In addition, the alginate matrix buffers the effect of mechanical disruption of the cells, which is the most reliable treatment, compared with freeze-thawing and enzymatic treatment with lysozymes. Our solution of dissolving alginate matrices with an acetate solution (Bashan and de Bashan, 2016), shown in this study, was the best choice to first release the cells from the immobilizing matrix and then expose them to the cell disruption treatment that follows. Mechanical disruption by beating with glass beads, combined with heat, improved isolation of nucleic acids, although an issue regarding the quality of the genetic material arose. Specifically, heating leads to partial disruption of the cell walls, over-releasing polysaccharides that inhibit downstream reactions, such as PCR (Tear et al., 2013). To address this specific problem, we used a strong cationic surfactant detergent (CTAB). Chaotropic agents, such as phenol, guanidine thiocyanate, and solvents yielded two isolation methods for each nucleic acid DNA and RNA.

In general, we found that the quantity (yield) and quality of nucleic acids changed significantly, depending mainly on growth conditions, length of incubation of the original culture, and to a lesser extent, the isolation method. Contrary to our expectations, the highest amounts of DNA and RNA were recovered from immobilized cells by either isolation procedures. A plausible explanation, yet to be proved, is that cells growing in highly clustered groups within polymeric matrices (Covarrubias et al., 2012) may undergo cell wall changes forced by congestion of the population, compared to free-living cells. Congestion made cell disruption easier after cells were released from the alginate beads. This theory is supported by our observations of cell disruption by bead-beating and heat-lysing one fraction of cells while others were weakened enough to increase their cell wall permeability and release considerable amounts of DNA (Fig. S1).

Immobilization showed similar effects on the quality of nucleic acids. Differences in the quality of DNA were more evident in the 260/230 absorbance ratio than the 280/260 ratio. Usually, "pure" nucleic acids have 260/280 and 260/230 absorbance ratios >1.8 (Sambrook and Russell, 2001). Our data of the 260/280 absorbance ratio indicated that both procedures were efficient at removing contaminants related to proteins. Absorbance curves for DNA extracts confirmed this result and also revealed a substantial noise in the absorbance at 220–240 nm, resulting in a low 260/230 absorbance ratio, especially in isolates using the Promega kit. Contaminants and extracting agents that absorb light at a wavelength ~230 nm include phenols, aromatic compounds, and carbohydrates (Wang and Stegemann, 2010). In our study, at least three out of four protocols used one of these reagents. The Promega kit does not specifically state the use of any of these. Since all samples exhibited contamination at similar absorbance, it is

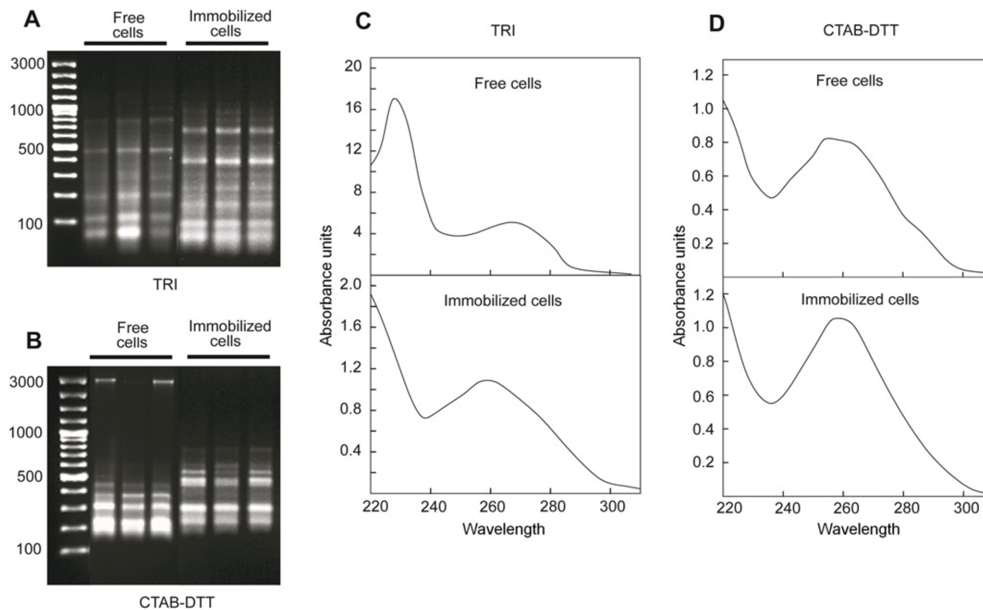

Fig. 4. Quality of total RNA extracted from *Chlorella sorokiniana* UTEX 2714 grown under two growth conditions and two isolation procedures. Cell conditions: Free cells and immobilized cells in alginate beads; RNA isolation procedures: TRI = RNA isolation procedure using TRI Reagent commercial product; CTAB-DTT = RNA isolation procedure modified from Thanh et al. (2009). A and B: Electrophoresis of RNA extracts in 1.5% native agarose gels, C and D: Spectrophotometric scan profiles acquired from Nanodrop. Each profile is representative of 12 at each growth condition. Agarose gels were stained with SYBR gold.

Table 2
Absorbance ratios of the RNA isolates extracted from *Chlorella sorokiniana* UTEX 2714 under two growth conditions and two isolation procedures.

Cell condition	260/280		260/230	
	TRI	CTAB-DTT	TRI	CTAB-DTT
Free cells	1.75 ± 0.02	2.09 ± 0.03	0.26 ± 0.01	0.96 ± 0.05
Immobilized cells	1.83 ± 0.03	2.02 ± 0.04	0.51 ± 0.08	1.19 ± 0.05

Average values ± Standard error (SE).
$n = 12$.
CTAB-DTT: RNA isolation procedure modified from Thanh et al. (2009).
TRI: RNA isolation procedure with Tri Reagent, commercial product.

possible that the extracts reflect also the abundance of other metabolites that are naturally produced by the microalgae. Tear et al. (2013) propose that extraction of genetic material from *Chlorella* species is more affected by the abundance of lipids rather than the rigidity of cell walls, and that this effect intensifies as the algal culture ages and lipid production increases. The strain of microalgae used in our study can accumulate significant amounts of lipids (de Bashan et al., 2002; Leyva et al., 2014, 2015). Hence, lipids along with other interfering metabolites, could explain the decreased yield of DNA observed at 72 h of incubation. Additionally, polysaccharides, the building blocks of alginate that immobilized the cells, may have another source of contamination that was detected at ~230 nm. In general, nucleic acids extracted by the Fawley-CTAB and CTAB-DTT procedures exhibited better quality DNA/RNA, defined by the 260/230 ratio. One of the advantages of the CTAB-based procedures is its versatility to precipitate nucleic acids and polysaccharides, depending on the ionic strength of the solution (Sambrook and Russell, 2001). In solutions of high ionic strength, CTAB will form complexes with proteins but will not precipitate nucleic acids. At low ionic strength, CTAB precipitates nucleic acids and acidic polysaccharides (Sambrook and Russell, 2001; Tan and Yiap, 2009). In our study, the ionic environment was favorable during isolation by the Fawley-CTAB procedure, but only at the lowest yield of DNA (72 h), since the highest yield of DNA was obtained by the Promega procedure in immobilized cells at 48 h. The low yield of nucleic acids isolated by the CTAB procedures might be explained by changes in the ionic environment of the extracts and by co-precipitation of nucleic acids with contaminants, such as polyphenols and polysaccharides that bind to nucleic acids during nucleic acid isolation (Japelaghi et al., 2011; Tan and Yiap, 2009).

In our study, carryover contaminants (from residual reagents and/or secondary metabolites) in the DNA extracts of immobilized or free cells did not limit amplification of nuclear 18S DNA and had only minor impact on the quality of the final PCR products. A version of our Promega procedure was recently used (Bashan et al., 2016) to isolate genomic DNA from free-living algal cultures to redefine the taxonomic identity of *Chlorella* UTEX 2714. In that study, extracts suitable for PCR were cleaned by using the commercial kit, followed by full sequencing of the 18S rDNA + ITS of the strain UTEX 2714. Genomic DNA extracted with both procedures from free and immobilized cells allowed us to also amplify the chloroplast gene of the carboxylase oxygenase (RuBisCO) large subunit (*rbcL* gene) from *C. sorokiniana* UTEX 2714 (Fig. S2).

Similar to other studies, cell disruption for RNA extraction was only achieved by freezing the microalgae pellet after centrifugation in liquid nitrogen and grinding the pellet using mortar and pestle (Tan and Yiap, 2009). In addition to the earlier discussion of larger amounts of nucleic acids obtained from immobilized cells, there were also clear differences in the quality and quantity of the isolated RNA obtained from each isolation method. The TRI Reagent procedure recovered larger amounts of RNA than CTAB-DTT in both free-living and immobilized cells. Higher yields of RNA with the TRI Reagent procedure are explained by the lower pH of the solution containing guanidinium thiocyanate, sodium acetate, phenol, and chloroform that efficiently separate RNA from DNA (Tan and Yiap, 2009). Similar to DNA, our RNA extracts were only affected by compounds other than proteins, as indicated by the low 260/230 absorbance ratios, mainly in TRI Reagent extracts. Alkaline extraction with CTAB-DTT and further precipitation with a high concentration of LiCl produced high quantity and quality RNA in the green microalga *Ankistrodesmus convolutus* (Thanh et al., 2009). Little information is available regarding RNA extraction directly from encapsulated cells. We found the CTAB-DTT procedure reliable for direct extraction of high quality RNA from immobilized cells, although it compromised the quantity of RNA. Wang and Stegemann (2010) isolated RNA from human cells in polysaccharide-based matrices. They found that CTAB extraction yielded significantly more RNA with higher purity than guanidine thiocyanate-based methods alone. This is in disagreement with

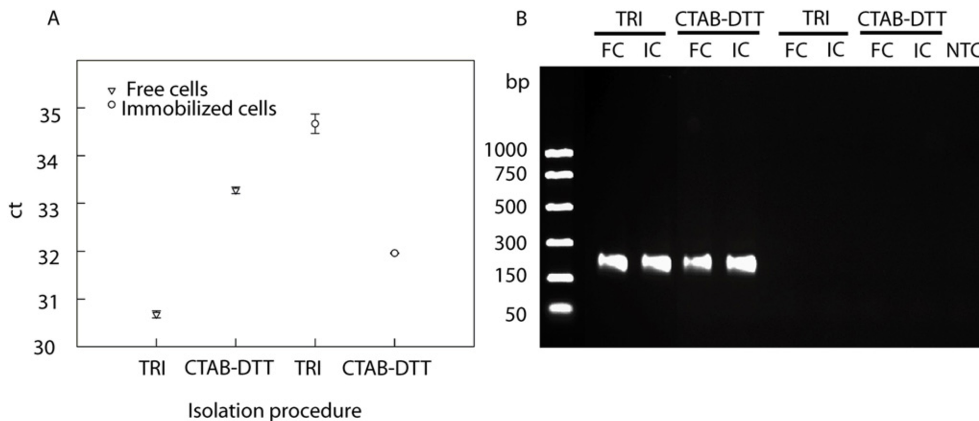

Fig. 5. Amplification of 18S rRNA of *Chlorella sorokiniana* UTEX 2714 obtained by RT q-PCR. Microalgae were grown under two growth conditions: Free cells (FC) and immobilized cells in alginate beads (IC). RNA isolation procedures: TRI = RNA isolation procedure, using TRI Reagent commercial product. CTAB-DTT = RNA isolation procedure modified from Thanh et al. (2009). A: Cycle threshold values of RT q-PCR of 18S cDNA, $n = 3$. B: Agarose gel electrophoresis analysis of RT q-PCR of the 192-bp 18S cDNA. Negative controls consist of RNA that is free of DNA. Agarose gel was stained with SYBR gold.

our general results, which may be explained by the presence and chemical complexity of the cell wall of the microalgae. They also found that even after purification of the RNA, using commercial columns, low 260/230 ratios were associated with contamination by residual polysaccharides from the matrices. Our results, and an additional source of noise that reduced 260/230 ratios in samples isolated by the TRI Reagent procedure, can be explained by the abundance of residual compounds contained in the TRI Reagent, such as phenol or guanidine thiocyanate that are absorbed at ~230 nm (Sambrook and Russell, 2001). This noise can be eliminated by column purification with commercial kits. This common strategy has recently allowed sequencing of cDNA libraries from mRNA isolated by the TRI Reagent method, resulting in the core transcriptome of *C. sorokiniana* 2714, where the full gene of the *rbcL* has been used in phylogenetic analysis (Bashan et al., 2016).

Even if extracts from the CTAB-DTT method exhibited higher purity than extracts of the TRI Reagent procedure, RNA isolates from free-living cells showed fewer bands on agarose gels, especially the upper bands that are expected to be the 28S and 18S rRNA, which are an indirect indicator of the population of mRNAs (Fig. 4A). DNA contamination was successfully eliminated by DNase treatment. However, the reduced integrity of RNA isolates from free-living cells and the CTAB-DTT procedure resulted in late cycle threshold values in RT-qPCR. This suggests lower recovering of the 18S rRNA in their RNA isolates. One of the reasons for late amplification could be the presence of PCR inhibitors, such as traces of phenol (Suslov and Steindler, 2005). Yet given the great variation in cycle thresholds for such an abundant gene, future studies are encouraged to extensively screen and select appropriate reference genes for quantitative RT-qPCR experiments (Fleige et al., 2006), especially of *Chlorella sorokiniana* strains proposed for biotechnological applications.

This study successfully standardized isolation methods for immobilized and planktonic cells of *C. sorokiniana*. Surprisingly, immobilization of microalgae favored the extraction of DNA and RNA, but has some negative ramifications on the quality the extracts. In terms of quantity, the modified procedure described in our study, including the Promega kit and pre-release of the cells from the polymeric matrix, was the most reliable procedure for DNA isolation and the modified procedure, using the commercial TRI reagent procedure, could be the choice for RNA isolation. However, we recommend a final step of column purification. This first report, analyzing the effect of immobilization on nucleic acid from immobilized microalgae cells, serves as the baseline for further studies on transcriptomics and functional genomics of *C. sorokiniana* strains that are under underway by our research group.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mimet.2017.02.005.

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