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A versatile method for simultaneous stable carbon isotope analysis of DNA and RNA nucleotides by liquid chromatography/isotope ratio mass spectrometry

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RATIONALE: Liquid chromatography/isotope ratio mass spectrometry (LC/IRMS) is currently the most accurate and precise technique for the measurement of compound-specific stable carbon isotope ratios ($^{13}C/^{12}C$) in biological metabolites, at their natural abundance. However, until now this technique could not be applied for the analysis of nucleic acids, the building blocks of the carriers of genetic information in living cells and viruses, DNA and RNA.
METHODS: Mixed-mode chromatography (MMC) was applied to obtain the complete separation of nine nucleotides (eight originating from DNA/RNA and one nucleotide (inosine monophosphate) that may serve as an internal standard) in a single run using LC/IRMS. We also developed and validated a method for DNA and RNA extraction and an enzymatic hydrolysis protocol for natural samples, which is compatible with LC/IRMS analysis as it minimizes the carbon blank. The method was used to measure the concentration and stable carbon isotope ratio of DNA and RNA nucleotides in marine sediment and in the common marine macro alga (*Ulva* sp.) at natural abundance levels as well as for ^{13}C-enriched samples.
RESULTS: The detection limit of the LC/IRMS method varied between 1.0 nmol for most nucleotides and 2.0 nmol for late-eluting compounds. The intraday and interday reproducibility of nucleotide concentration measurements was better than, respectively, 4.1% and 8.9% and for $\delta^{13}C$ measurements better than, respectively, 0.3‰ and 0.5‰. The obtained nucleic acid concentrations and nucleic acid synthesis rates were in good agreement with values reported in the literature.
CONCLUSIONS: This new method gives reproducible results for the concentration and $\delta^{13}C$ values of nine nucleotides. This solvent-free chromatographic method may also be used for other purposes, such as for instance to determine nucleotide concentrations using spectrophotometric detection. This sensitive method offers a new avenue for the study of DNA and RNA biosynthesis that can be applied in various fields of research. Copyright © 2014 John Wiley & Sons, Ltd.

The nucleic acids, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), are both essential for all forms of life. Nucleic acids are among the most important biopolymers together with proteins and carbohydrates and are abundantly present in all organisms, where they serve to encode, transmit and express genetic information.[1] Nucleic acids are composed of nucleotide monomers which consist of a phosphate group, a pentose sugar and a purine or pyrimidine base. The genetic information is contained in the sequence of these nucleotides.[2] In addition to being the building blocks of DNA and RNA, nucleotides are also crucial components of metabolic processes, for instance in the form of ATP, which serves as the major energy carrier in organisms.[3] Nucleic acids are also essential intermediates in the global carbon cycle.[4]

Compound-specific stable isotope techniques are state-of-the-art for the study of the carbon cycle, because they provide information about the concentrations, and the source and turnover rates of the compounds of interest.[5] The most common approach to determine the natural abundance and low enrichment $^{13}C/^{12}C$ ratios in specific metabolites is compound-specific stable isotope analysis (CSIA) by isotope ratio mass spectrometry (IRMS).[6] The main advantage of this technique is that the various compounds can be separated before analysis without interference from impurities. Separation of the compounds can be performed by either gas chromatography (GC) or liquid chromatography (LC). However, many of the components of interest in biological systems such as RNA and DNA nucleotides are polar and/or polymeric and therefore LC is the preferred chromatographic separation method.

The determination of DNA synthesis rates by using stable isotope ratios in combination with LC and Chemical Reaction Interface Mass Spectrometry (CRIMS) has been described.[7,8] Auclair and coworkers[9] developed a method that uses a liquid chromatograph coupled to a quadrupole mass spectrometer in order to measure the ^{13}C enrichment of thymine incorporated into DNA. However, because of the relatively low accuracy at low enrichment of conventional mass spectrometry (MS), highly enriched compounds are required for reliable measurement of isotope ratios.[8,10] In addition, extensive incorporation of the labeled substrate is needed, eventually leading to

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excessive incubation times. The results obtained with this approach may therefore not reflect the actual environmental metabolism and hampers the accurate monitoring of ^{13}C incorporation in DNA or RNA. Chen and Abramson[11] described a method to determine DNA synthesis by analyzing ^{13}C incorporation into the corresponding nucleosides based on the use of high-performance liquid chromatography chemical reaction interface IRMS (HPLC/CRI/IRMS). Although this approach is highly sensitive for measuring isotopic enrichment, it involves complex instrumentation that is not commercially available.

LC/IRMS is a promising technique for the compound-specific ^{13}C analysis of DNA and RNA, because of its ability to measure $\delta^{13}C$ values at natural abundance as well as after isotope labeling in selected metabolites. For instance, methods for carbohydrate, amino acid and short-chain organic acid analysis have been successfully implemented and used to study carbon cycling in microphytobentic communities in intertidal sediments and provided valuable information on bacteria-diatom interactions.[12] Also in other research fields, a substantial number of applications to study metabolites have been published, emphasizing the power and robustness of LC/IRMS.[13–20]

The design of the LC/IRMS interface involves a number of analytical constraints.[12,26] The current design is not compatible with organic and other carbon-containing eluents, as the continuous oxidation of the eluent in the reactor unit would create a very high CO_2 background. Bleeding of the analytical column should also be minimal for the same reason. In addition, to obtain accurate isotopic measurements, the components should be baseline separated. Considering all restrictions, the main challenge to performing CSIA of DNA and RNA nucleotides by LC/IRMS is to select a suitable column and the right conditions to separate all eight DNA and RNA nucleotides in one single run. Subsequently, a commonly used method to simultaneously extract DNA and RNA in conjunction with a method to hydrolyze the extracted nucleic acids to nucleotides had to be adapted to determine concentration and $\delta^{13}C$ values by LC/IRMS. All the adaptations were made to comply with the analytical constraints of the design of the LC/IRMS interface.

A first attempt to analyze RNA nucleotides by LC/IRMS was made by MacGregor *et al.*[21] McCullagh[22] showed that polar molecules, such as a number of DNA-related nucleosides, can be retained and eluted from mixed-mode columns. However, a complete developed LC/IRMS method to perform CSIA for nucleic acids was not available. The aim of the present study was to develop a method to simultaneously measure concentrations and $\delta^{13}C$ values in DNA and RNA nucleotides and to demonstrate its applicability for the study of DNA and RNA synthesis in intertidal marine sediments as well as in a macro alga.

EXPERIMENTAL

Chemicals and standards

Deoxyadenosine monophosphate was purchased from MP Biomedicals (Eindhoven, The Netherlands). All other reagents were purchased from Sigma (St, Louis, MO, USA) and were of analytical grade except for the nucleotide standards (purity >95%). Detailed information, such as name abbreviations, structural formulae, extinction coefficients, carbon content and molecular weights for all the nucleotides, is listed in Table 1. Nuclease P1, obtained as a lyophilized powder, was dissolved in 50 mM potassium phosphate buffer, pH 5.8, and stored at 4°C for a maximum of 3 weeks. Freshly prepared Milli-Q water (18.2 MΩ, DOC free; Millipore, Bedford, MA, USA) was used in all experiments.

The exact molecular masses of the purchased nucleotide standards were not known since the standards contained unspecified amounts of crystalline water and sodium. Hydrous molecular masses were calculated by two methods: (i) the carbon content of the individual nucleotides was determined by elemental analyzer/isotope ratio mass spectrometry (EA/IRMS) and (ii) the concentration of individual prepared stock solutions was determined via UV–VIS absorption spectrophotometric analysis.

The carbon content was determined by a Flash EA 1112 Series elemental analyzer (EA) coupled via a Conflo III interface to a Delta V Advantage isotope ratio mass spectrometer (all from Thermo Fisher Scientific, Bremen, Germany).[23] The molecular mass (MM) of the nucleotide (g/mol) was defined as $MM_{NTD} = MM\ C_{NTD} * 100/C\ \%$, where MM C_{NTD} denotes the molecular mass of carbon in the nucleotide as derived from the formulae in Table 1 and C % is the carbon content determined by EA/IRMS.

UV–VIS, using Beer's Law in combination with extinction coefficients (published by Cavaluzzi and Borer[24] and Clonis and Lowe[25]), was used as an alternative way to determine the hydrous molecular masses of the purchased nucleotide standards. Stock solutions (~10 mM in Milli-Q water) of the individual nucleotide standards were prepared, and subsequently diluted (in 100 mM sodium phosphate buffer, pH 7.0) to obtain an absorbance within the linear range of the spectrophotometer (absorbance unit <1.5), resulting in a series of five different nucleotide concentrations in the range of 0–100 µM. Each individual nucleotide concentration was measured in triplicate. The molecular mass of each individual nucleotide was calculated from the measured concentration and the weight of the stock solution.

LC/IRMS analysis of DNA and RNA nucleotides

High-performance liquid chromatography (HPLC) was carried out on a Surveyor system consisting of a HPLC pump (MS Pump Plus) and an Autosampler Plus autoinjector (all from Thermo Fisher Scientific), fitted with a PrimeSep D guard and mixed-mode analytical column (4.6× 150 mm, particle size 5 µm, 100 Å; Sielc, Prospect Heights, IL, USA) and eluted at 500 µL min^{-1} isocratically with 10 mM H_2SO_4, pH 2. The eluent was degassed in an ultrasonic bath for 15 min and further degassed with helium during analysis. 'No-Ox' tubing (1/8"× 1.5" mm; Sococchim, Lausanne, Switzerland) was used to connect the eluent bottles to the pump to prevent atmospheric gases from re-entering the solvents. All pump heads were rinsed at least once a day. An in-line filter of 2 µm (Vici, Schmidlin Labor, Neuheim, Switzerland) was placed after the LC column to prevent particles from entering the interface.

The HPLC system was coupled to the IRMS instrument by a LC isolink interface (Thermo Fisher Scientific) first described by Krummen *et al.*[26] The Isolink interface performs wet oxidation

Table 1. Characteristics of purchased DNA and RNA nucleotides. Calculated hydrous molecular masses and %C data are based on triplicate analysis. Coefficients of variation (CV) are expressed in %

							Calculated Molecular Masses (hydrous)						
			MM*	ε260 (pH 7)***	%C		MM via EA/IRMS			MM via UV–VIS			Difference MM
Nucleotide		Formula	g/mol	L/mmol /cm	****	SD	g/mol	SD	CV%	g/mol	SD	CV%	%
Cytidine monophosphate	CMP	$C_9H_{12}N_3Na_2O_8P.yH_2O$	367.2	7.07	21.6	0.0	501	1	0.2	504	5	0.9	0.5
Adenosine monophosphate	AMP	$C_{10}H_{12}N_5Na_2O_7P.yH_2O$	391.2	15.02	23.8	0.0	504	1	0.2	518	2	0.4	2.6
Guanosine monophosphate	GMP	$C_{10}H_{12}N_5Na_2O_8P.yH_2O$	407.2	12.08	22.2	0.2	541	6	1.1	538	6	1.0	0.6
Uridine monophosphate	UMP	$C_9H_{11}N_2Na_2O_9P$	368.2	9.66	21.8	0.1	496	2	0.4	504	8	1.6	1.7
Deoxycytidine monophosphate	dCMP	$C_9H_{12}N_3Na_2O_7P.yH_2O$	351.2	7.10	21.9	0.2	494	5	1.0	463	6	1.2	6.6
Deoxyadenosine monophosphate	dAMP	$C_{10}H_{12}N_5Na_2O_6P.yH_2O$	375.2	15.06	26.6	0.2	451	4	0.9	470	5	1.0	3.9
Deoxyguanosine monophosphate	dGMP	$C_{10}H_{14}N_5O_7P.xNa.yH_2O$	347.2**	12.18	24.4	0.1	493	2	0.5	506	4	0.8	2.7
Deoxythymidine monophosphate	dTMP	$C_{10}H_{13}N_2Na_2O_8P.yH_2O$	366.2	8.56	27.4	0.1	438	2	0.4	447	6	1.3	1.9
Inosine monophosphate	IMP	$C_{10}H_{11}N_4Na_2O_8P.yH_2O$	392.2	12.3 (250 nm)	22.1	0.1	544	3	0.6	563	4	0.6	3.4

*anhydrous based.
**free acid based.
***Cavaluzzi and Borer,[24] Clonis and Lowe.[25]
****determined via EA/IRMS analysis of the hydrated compound.

of organic compounds with peroxodisulfate under acidic conditions. The CO_2 that is produced from the oxidation was separated from the mobile phase in a capillary gas exchanger flushed with helium gas, was then dried, and subsequently entered the ion source of the mass spectrometer in a helium stream via an open split interface. The temperature of the oxidation reactor was set at 99.9°C. The flow rates of the acid (1.5 M H_3PO_4) and oxidant reagent (0.7 M $Na_2S_2O_8$) were each 50 µL/min.

Isotopic ratio measurements were carried out on a Delta V Advantage isotope ratio mass spectrometer. The LC/IRMS system and data collection were controlled using Isodat 2.5 SP 1.13 software (Thermo Fisher Scientific). To calibrate the system, two pulses of about 20 s each of CO_2 reference gas were admitted into the inlet of the mass spectrometer at the beginning of a run. The reference gas was regularly calibrated against phthalic acid (Schimmelman, Indiana University, Bloomington, IN, USA) with a $\delta^{13}C$ value of -27.21 ± 0.02‰.

Peak identification was based on retention times obtained from external standards. Concentration measurements were based on the peak areas of the separated compounds and calibrated against external standards. The injection volume was 50 µL for sediment samples and 10 µL for samples from the macro alga. Intraday (within a day) and interday (between days) reproducibility of the nucleotide concentration and $\delta^{13}C$ values by LC/IRMS were assessed by replicate analysis ($n = 8$ for intraday precision and $n = 3$ for interday precision) of the peak areas and $\delta^{13}C$ values of all standards (1000 µM) on, respectively, one day and three different days in a 4-week time period.

The carbon content and isotopic composition of total organic carbon (TOC) were analyzed by EA/IRMS. All samples were analyzed in triplicate.

Determination of carbon isotopic composition of standards

The carbon isotopic composition of individual nucleotide standards was determined by three different techniques. First, the $\delta^{13}C$ values were determined by EA/IRMS. Second, they were determined by flow injection analysis IRMS (FIA/IRMS): via direct injection into the flow path of the interface of the LC/IRMS system, the isotopic composition of individual nucleotides can be determined without using column separation.[16] Like EA/IRMS, FIA/IRMS measures bulk $\delta^{13}C$ values. The difference between the two techniques is that for FIA/IRMS the nucleotides need to be dissolved in Milli-Q water while for EA/IRMS they are applied in solid form. Third, the $\delta^{13}C$ values were determined by LC/IRMS. All values for each technique were determined in triplicate.

Environmental samples

Intertidal marine sediment and the marine macro alga (*Ulva* sp.) were collected from the Eastern Scheldt estuary (The Netherlands) and analyzed for their DNA/RNA nucleotide isotopic composition. Six sediment cores (7 cm i.d.) and *Ulva* sp. were collected. The sediment was covered with a thin brown mat of microalgae mainly composed of diatoms. Three sediment cores were labeled by adding 1 mL of a 10 mM $NaH^{13}CO_3$ (99% ^{13}C, Isotec, Stein, The Netherlands) solution to the surface of the sediment and were incubated for 4 h at ambient conditions of temperature and light. The $NaH^{13}CO_3$ solution was prepared in artificial seawater (Ca^{2+}- and Mg^{2+}-free) with a salinity of 30 ‰. Triplicate unlabeled cores were also processed. Subsequently, the upper 1.5 cm layer of the labeled and unlabeled sediment cores was sampled and thoroughly mixed. The *Ulva* sp. was labeled under the same conditions. All samples were directly frozen and stored at −80°C until analysis.

Sample preparation

DNA/RNA extraction

DNA and RNA were co-extracted from the samples according to Griffiths *et al.*[27] and Hurt *et al.*,[28] with modifications. Briefly, 5–10 g (wet weight) of sediment sample and 1 g (wet weight) of *Ulva* sp. were added to 3 mL denaturing solution (4 M guanidine isothiocyanate, 10 mM Tris–HCl (pH 7.0), 1.0 mM EDTA, 0.5% w/v 2-mercaptoethanol), vortexed for 3 min, followed by addition of 20 mL extraction buffer (100 mM sodium phosphate (pH 7.0), 100 mM Tris–HCl (pH 7.0), 0.1 mM EDTA (pH 8.0), 1.5 M NaCl, 1% w/v hexadecyltrimethylammonium bromide (CTAB) and 2% *N*-lauroyl-sarcosine), incubated at 65°C for 30 min with gently manual mixing every 10 min and centrifuged at 1800 *g* for 10 min at 4°C. The supernatants were transferred into a 50 mL tube on ice containing 20 mL aliquots of phenol/chloroform/isoamyl alcohol (25:24:1, pH 6.7), manually mixed for 10 min and centrifuged at 5000 *g* for 30 min at 4°C. The aqueous phase was recovered and residual phenol was removed by an equal volume (approximately 20 mL) of chloroform/isoamyl alcohol (24:1) followed by centrifugation at 5000 *g* for 20 min at 4°C. Subsequently, nucleic acids were precipitated from the aqueous layer with 1 volume of PEG-8000, 10% w/v, incubated for 12 h at 4°C, followed by centrifugation at 5000 *g* for 20 min at 4°C. Pelleted nucleic acids were washed at least 4 times with ice-cold 70% (v/v) ethanol and air-dried prior to dissolution in 60 µL Milli-Q water. The extracts were stored at −80°C until analysis. The DNA and RNA purity and approximate quantity were determined by a model ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Hydrolysis of DNA/RNA

The extracted DNA and RNA were hydrolyzed by Nuclease P1, which cleaves both RNA and single-stranded DNA into 5′-mononucleotides. The protocol was a modification of the method of Shimelis and Giese.[29] DNA/RNA (60 µL, 19–50 µg total DNA/RNA) was denatured at 95°C for 15 min and treated with 80 µL 50 mM potassium phosphate (pH 5.8), 20 µL 30U/mL Nuclease P1 (in 50 mM potassium phosphate buffer, pH 5.8), and 30 µL Milli-Q water. The sample tube was capped and kept for 1 h at 37°C. By centrifugal ultrafiltration (NanoSep, 3 kDa, Pall Life Sciences, Ann Arbor, MI, USA) the enzyme was removed from the sample. Shortly before use the applied filters were washed 8 times with 200 µL Milli-Q water and centrifuged at 10 000 *g* for 5 min. Subsequently, together with the sample a known amount of internal standard (10 µL, 10 mM IMP) was applied in order to determine the recovery of the ultrafiltration step. All data were corrected for recovery. After centrifugal ultrafiltration of the sample (10 000 *g* for 5 min) the filter was rinsed with 2 aliquots

of 50 µL Milli-Q water. The sample and the rinse filtrate were pooled, freeze-dried and dissolved in 100 µL Milli-Q water before LC/IRMS analysis.

Calculations

The stable carbon isotope ratios are reported in the delta-notation:

$$\delta^{13}C\ (‰) = \left(R_{sample}/R_{VPDB}\right) - 1) \times 1000$$

where R_{sample} and R_{VPDB} denote the $^{13}C/^{12}C$ ratio in the sample and the international standard, Vienna Pee Dee Belemnite (for carbon $R_{VPDB} = 0.0111802 \pm 0.0000009$), respectively.

For metabolic studies it is more convenient to calculate the absolute amount of ^{13}C incorporated into different carbon pools above background. This value is expressed as excess ^{13}C and is calculated from the $\delta^{13}C_{sample}$ value as:

$$\text{excess}^{13}C\ \left(\text{mol}^{13}C\ g^{-1}DW\right) = \left[\left(\frac{(\delta^{13}C_{sample}/1000+1)\times R_{st}}{(\delta^{13}C\text{sample}/1000+1)\times R_{st}+1}\right) - \left(\frac{(\delta^{13}C_{background}/1000+1)\times R_{st}}{(\delta^{13}C_{background}/1000+1)\times R_{st}+1}\right)\right] \times C_{sample}$$

where $\delta^{13}C_{background}$ denotes the $\delta^{13}C$ value of the unlabeled sample and C_{sample} denotes the pool size in mol of carbon per gram of dry weight sample (mol C g^{-1} DW).

The synthesis rates of DNA and RNA are expressed in pmol $^{13}C/h/g$ DW and calculated by the sum of the excess values of the individual nucleotides, divided by the incubation time (4 h).

RESULTS AND DISCUSSION

Chromatography

Mixed-mode chromatography (MMC) was applied in order to obtain complete separation of the nine nucleotides (eight originating from DNA/RNA and IMP that was used as internal standard). The advantage of the MMC phase over other stationary phases is that interactions on the column are multiple controllable and effective under fully aqueous conditions. Therefore, this phase lends itself very well to the chromatographic niche created by the constraints of the current design of the liquid interface of the LC/IRMS system.[22]

The applied Primesep D column is a bimodal column using two retention mechanisms: reversed-phase and anion-exchange separation. The retention time of the analytes was controlled by ion-exchange interaction and the ionization state of the stationairy phase (which can be adjusted by changing the pH of the mobile phase) and hydrophobic interactions of the nucleotide and the stationairy phase. A concentration of 10 mM H_2SO_4 (pH 2) was found to be optimal for the separation of the eight nucleotides of DNA and RNA and the internal standard (IMP) (Fig. 1(A)). All the nucleotides were completely baseline-separated. In addition, the chromatograms from the extracted and hydrolyzed sediment samples are straightforward and simple

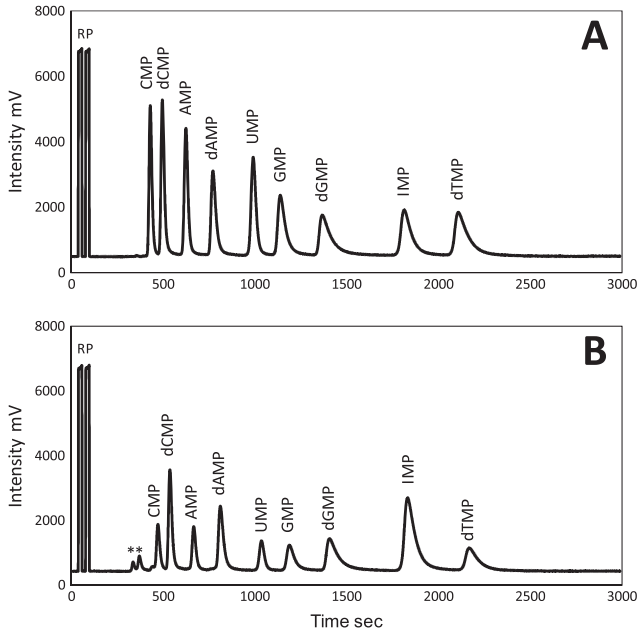

Figure 1. (A) LC/IRMS chromatogram showing the separation of DNA and RNA nucleotides standard mixture on the PrimeSep D column eluted isocratically with 10 mM H_2SO_4, pH 2 eluent. Injected was 10 µL, 1000 µM. (B) Chromatogram of an extracted and hydrolyzed intertidal sediment sample analyzed for DNA and RNA nucleotides. RP = reference peaks. * = impurities originating from the filter used in the hydrolysis protocol. Injected was 50 µL.

(e.g. Fig. 1(B)), showing excellent separation of the compounds of interest with minimal impurities. An impurity, originating from the filter used at the hydrolysis step, elutes at the beginning of the chromatogram. The concentration of this impurity is low and it does not interfere with the nucleotide analysis.

Although the chromatographic method was developed to separate nucleotides using LC/IRMS, it has also been applied successfully by us in traditional HPLC using UV spectrophotometric detection at 260 nm to analyze nucleotide concentrations with the advantage of a water-based, solvent-free method (data not shown).

Stable carbon isotope and concentration determination of nucleotide standards

The molecular masses (MMs) of the purchased hydrous nucleotides were determined in triplicate by two different methods: EA/IRMS and UV–VIS (Table 1). The calculated molecular masses were in good agreement for all nucleotides and the differences between the two methods were less than 6.6%. Differences between the two methods are probably caused by the different approach in analysis. For instance, impurities, detectable by EA/IRMS, will underestimate the MM, while this same impurity may not detectable by UV/VIS and thus will have no effect on the UV/VIS-determined MM, and vice versa. Although the EA/IRMS method may be slightly more precise, we conclude that both methods determine the MMs of the individual nucleotides accurately and may be used to prepare the calibration standard stock solutions.

A range of concentrations of nine standard nucleotides was injected to determine detection limits and linearity. The peak areas were highly linear with all concentrations tested (20 to 4000 µM (equals 200 pmol to 40 nmol nucleotide) injected with a 10 µL loop) with an R^2 higher than 0.9994 (data not shown), indicating that the method is suitable to determine nucleotide concentrations. Over the same concentration range the stable carbon isotope ratios remained within acceptable limits (standard deviation (SD) between replicate $\delta^{13}C$ values <0.5 ‰) between 1.0 nmol (110 ng C) nucleotide injected for most components and 2.0 nmol (250 ng C) for late-eluting nucleotides (GMP, dGMP and dTMP) and up to the highest concentrations (40 nmol) tested for all nucleotides. Figure 2 depicts the effect of the injected amount of two nucleotides (GMP and UMP) on the determined $\delta^{13}C$ values. Other nucleotides gave similar results. Typically, a peak height of 500 mV or more was required for accurate isotope ratio analysis. Below 500 mV substantial deviations from the expected isotopic ratio were found, which could presumably be attributed to problems with the baseline correction. With a 10 µL loop a detection limit of 100 to 200 µM could be reached. By using a 50 µL loop the sensitivity could be increased and a detection limit of 20 to 40 µM could be reached, providing ample scope for the carbon isotope analysis of nucleotides in natural materials.

As illustrated in Table 2, the intraday and interday precision, measured on respectively one day (n = 8) and on three different days in a 4-week period (n = 3), was good for concentration measurements and excellent for the isotopic analysis of all nucleotide standards. For concentration measurements the intraday precision was better than 4.1% and the interday precision was better than 8.9%. For $\delta^{13}C$ measurements the intraday and interday precision was better than 0.3‰ and 0.5‰, respectively. Moreover, the column was stable and we analyzed numerous standards and samples for more than 1 year without loss of performance.

Unfortunately, we were unable to determine the absolute accuracy of the $\delta^{13}C$ nucleotide analysis since there are no certified standards available. Therefore, we compared the $\delta^{13}C$ values as determined by LC/IRMS, FIA/IRMS and traditional EA/IRMS for all nucleotide standards in order to determine the reliability of the LC/IRMS data (Fig. 3). The carbon isotope ratios determined by FIA/IRMS generally corresponded well with the data obtained from EA/IRMS analyses, but for some nucleotides there was a small offset (Fig. 3(A)). The nucleotides were dissolved in Milli-Q water when analyzed by FIA/IRMS and the observed offset could probably be attributed to a ^{13}C-depleted carbon blank in the Milli-Q water. Unfortunately, the amount of carbon in this blank was too low (about 1 nmol C) for accurate $\delta^{13}C$ measurements and as a result no blank corrections could be made to the FIA/IRMS data. Subsequently, carbon isotope ratios determined by LC/IRMS were plotted against EA/IRMS-determined $\delta^{13}C$ values of individual nucleotides (Fig. 3(B)). More than half of the $\delta^{13}C$ nucleotide values determined by LC/IRMS showed a positive offset. This was probably because the commercial nucleotide standards contained a ^{13}C-depleted impurity, leading to an offset as the EA/IRMS method is not compound-specific. To conclude, concentrations and stable isotope ratios of the nine nucleotides (of which eight represented DNA and RNA) were analyzed reproducibly and accurately by LC/IRMS.

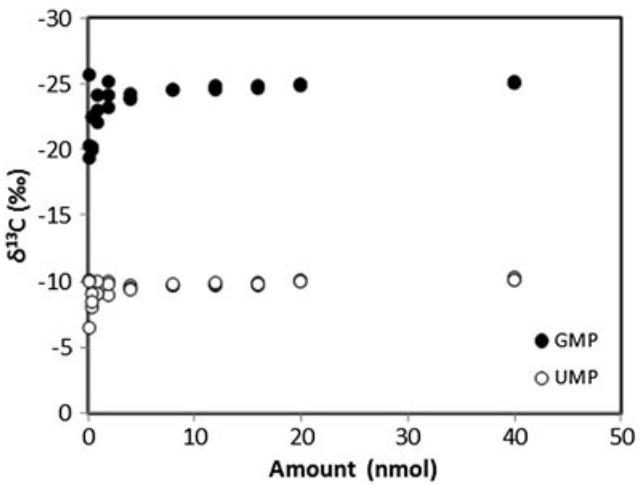

Figure 2. Effect of injected amount (0.2–40 nmol) of nucleotide on the stable isotope ratio of guanosine monophosphate (GMP) and uridine monophosphate (UMP). A 10 µL injection loop was used. Other nucleotides gave similar results.

Testing the sample preparation protocol

The phenol/chloroform extraction method was adapted in order to simultaneously extract DNA and RNA from environmental samples.[27,28] The ratio of absorbance at 260 and 280 nm as measured by the Nanodrop ND-1000 spectrophotometer was used to determine the purity of DNA and RNA (data not shown). A ratio (260/280) of ~1.8 indicates high-purity nucleic acids and implies a successful isolation of the nucleic acids (ND-1000 user manual). A lower ratio indicates the presence of impurities such as proteins, phenol or other contaminants that absorb strongly near 280 nm and could influence the LC/IRMS $\delta^{13}C$ determination. The 260/280 ratios of the extracted nucleic acids from the sediment samples and the *Ulva* sp. were 1.98 ± 0.06 and 2.17 ± 0.05, respectively, and these were considered adequate. It was determined experimentally that the amount of extracted sediment in this protocol should not exceed 10 g; otherwise, the purity of the extracted DNA and RNA decreased (data not shown). Additional rinsing of the DNA/RNA pellet with 70% ethanol and subsequent freeze-drying were necessary to avoid interference from residual carbon-containing organics. Usually, water treated with diethylpyrocarbonate (DEPC) is used for handling RNA, in order to reduce the risk of RNA degradation by RNAse. However, DEPC treatment contributes approximately 20 ng C to the protocol blank.[30] Therefore, we decided to use freshly prepared Milli-Q instead.

In order to decrease the protocol carbon blank the original hydrolysis method of Shimelis and Giese[29] was modified in two ways: the buffers (TRIS and acetate) were replaced by phosphate buffers and zinc chloride was replaced by potassium chloride. The first modification was made in order to decrease the protocol carbon blank. The second was made because zinc precipitates with the phosphate buffer or with the phosphoric acid in the LC/IRMS interface, and this will cause clogging of the system. Nuclease P1 is a zinc-dependent enzyme; however, it is possible to replace zinc by potassium.[31] Whereas zinc

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Table 2. Intraday and interday variability (precision) of concentration measurements (1000 µM, 10 µL injection) and $\delta^{13}C$ values of DNA and RNA nucleotides and one internal standard (IMP) analyzed by LC/IRMS. Values are the mean, standard deviation (SD) and coefficients of variation (CV) of replicate measurements (n = 8) of peak areas and $\delta^{13}C$ values. The intraday variability is the mean of eight replicates measured in one day. The interday precision was determined by the average of three replicate measurements averaged for three different days (days 1, 5 and 30) within the period of a month

	Intraday variability			Interday variability			Interday variability		Interday variability	
NTD	Area	SD	CV%	Area	SD	CV%	$\delta^{13}C$ ‰	SD	$\delta^{13}C$ ‰	SD
CMP	90.3	1.9	2.1	88.9	2.0	2.3	−11.5	0.1	−11.5	0.0
AMP	104.5	2.8	2.7	99.6	6.6	6.6	−8.9	0.1	−8.9	0.1
GMP	94.7	1.6	1.7	96.0	4.0	4.2	−24.7	0.3	−24.8	0.3
UMP	101.2	4.2	4.1	101.0	5.3	5.2	−10.7	0.2	−10.3	0.1
dCMP	107.3	3.4	3.1	101.4	6.8	6.7	−18.4	0.1	−18.2	0.1
dAMP	90.0	2.6	2.9	88.7	4.9	5.5	−17.7	0.3	−17.8	0.3
dGMP	90.5	2.5	2.8	93.5	4.3	4.6	−21.1	0.3	−20.8	0.5
dTMP	107.8	3.5	3.3	106.8	7.7	7.2	−19.1	0.2	−19.4	0.4
IMP	93.8	1.9	2.0	86.7	7.7	8.9	−14.9	0.3	−14.9	0.4

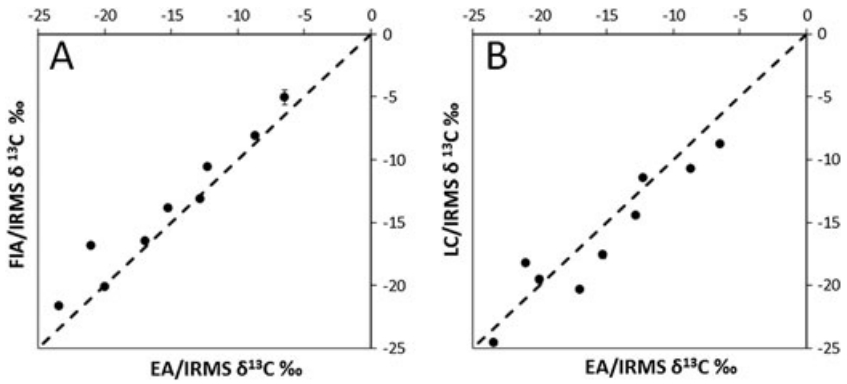

Figure 3. Comparison of stable isotope ratios of individual nucleotides by FIA/IRMS versus traditional EA/IRMS (A) and LC/IRMS versus traditional EA/IRMS (B). The y = x line is indicated in both figures. Stable isotope data are the average of three replicate analyses.

chloride increases the activity of Nuclease P1 by 248%, potassium chloride results in an increase of 172%.[31] The optimum amount of enzyme and the incubation times were tested using calf thymus DNA. An incubation time of more than 60 min was necessary to digest >88% of the DNA to its nucleotides and 20 µL (30 U/mL) Nuclease P1 was optimal (Figs. 4(A) and 4(B)). Long incubation times should be avoided because Nuclease P1 may digest the nucleotides.[29] However, an incubation time of up to 240 min, as applied here, showed no evidence for nucleotide losses (Fig. 4(A)). Using more than 20 µL 30 U/mL Nuclease P1 resulted in a decrease in the amount of nucleotides, which may have been caused by the digestion of the nucleotides by the excess nuclease (Fig. 4(B)). In order to prevent these undesirable digestions, the DNA/RNA-to-enzyme ratio was kept constant, and after the hydrolysis reaction Nuclease P1 was immediately removed by centrifugal ultrafiltration. The ultrafiltration filters had to be washed with Milli-Q water in order to obtain a blank that was acceptable (see Fig. 1(B); peak intensity of impurities at retention time ~350 s < 1000 mV) and that did not interfere with the nucleotide analysis.

Nuclease P1 solution stored at 4°C is stable for 3 weeks. When required, Nuclease P1 can be made stable for a longer period of time by immobilization on chitosan nanoparticles.[32] For a batch of samples, the quality of Nuclease P1 was always checked by its ability to digest calf thymus DNA. We found a G-C and A-T content of calf thymus DNA of 41.6 ± 0.1 mol % and 58.4 ± 0.1 mol % (n = 3), respectively, which is in agreement with published values (41.9 mol % G-C and 58.1 mol % A-T[33]). The recovery of the Nuclease P1-digested DNA was 88 ± 5% for an incubation time of 60 min. The recovery after the freeze-drying and centrifugal ultrafiltration steps was 96.7 ± 0.7% for the freeze-drying treatment and 70.0 ± 0.9% for the ultrafiltration treatment. There are various other methods to hydrolyze DNA, but they were not considered, because severe hydrolysis conditions in combination with long incubation times may result in the deamination of nucleotides and other undesirable side effects.[34] We conclude that the enzymatic hydrolysis with Nuclease P1 is a practical and reliable method for simultaneous digesting of nucleic acids into quantifiable nucleotides.

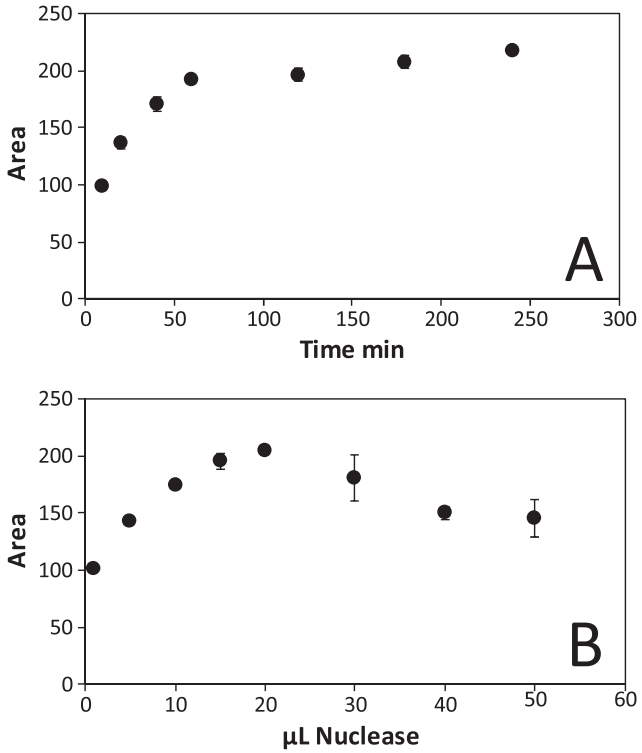

Figure 4. (A) Release of DNA nucleotides as a function of hydrolysis time by 20 µL, 30U/mL Nuclease P1 in 50 mM potassium phosphate buffer, pH 5.8, at 70°C. A fixed concentration of 1.0 g/L calf thymus DNA was applied. (B) Release of DNA nucleotides as a function of varying amount of 30 U/mL Nuclease P1 in 50 mM potassium phosphate buffer, pH 5.8 at 70°C. A fixed concentration of 1.0 g/L calf thymus DNA was applied.

Stable carbon isotope analysis of DNA and RNA in environmental samples

We analyzed a diatom mat from an intertidal mudflat and a macro alga for DNA and RNA nucleotide concentrations (Table 3) and stable carbon isotope composition (Table 4). The measured concentrations of DNA and RNA (coefficient of variation (CV) better than 26%) in the studied samples were in good agreement with published values.[35–37] During the 4 h of *Ulva* sp. labeling the DNA concentration decreased by 50%, which was probably caused by the length of air exposure, which desiccated the *Ulva* sp. and made it prone to partial sun bleaching. The reproducibility of the measurement of stable isotope ratios for the individual nucleotides in the sediment and in *Ulva* sp. was excellent at natural abundance (better than SD ± 0.7 ‰ (n = 3)).

For the sediment the $^{13}C/^{12}C$ ratios of the individual nucleotides were higher than the total organic carbon ratios (Table 4). In the case of *Ulva* sp., the $\delta^{13}C$ values for the DNA nucleotides were typically lower and those for the RNA nucleotides were typically higher than the $\delta^{13}C$ values of the total organic carbon. An exception is the *Ulva* sp. DNA nucleotide dGMP, which was ^{13}C-enriched in comparison with total organic carbon. The variation in individual $\delta^{13}C$ nucleotide values was considerable. The pool-size averages of the $\delta^{13}C$ values for DNA and RNA nucleotides were, respectively, −13.8 ± 0.1 and −11.3 ± 0.1‰ for the marine

Table 3. DNA and RNA nucleotide concentrations as detected by LC/IRMS in intertidal marine sediment and in a macro alga (*Ulva* sp.) in isolated DNA and RNA, hydrolyzed enzymatically by Nuclease P1. The total organic carbon (TOC) content was determined by EA/IRMS. DNA and RNA nucleotide concentrations and standard deviation (SD) are the average of three replicate samples. Coefficients of variation (CV) are expressed in %

		RNA nucleotides				DNA nucleotides								
Sample		CMP	AMP	GMP	UMP	dCMP	dAMP	dGMP	dTMP	RNA	DNA	RNA/DNA	TOC
Sediment	nmolC/g DW	75	58	63	41	98	135	139	84	237	456	0.53	287751
	SD	16	5	10	4	19	13	18	8	7	55	0.08	3576
	CV%	22	9	16	11	19	10	13	10	3	12	15	1
Sediment labeled	nmolC/g DW	81	66	60	45	103	142	143	65	252	448	0.61	287286
	SD	13	16	12	7	10	1	17	12	48	19	0.06	12045
	CV%	16	24	20	17	10	1	12	18	19	4	10	4
Ulva	nmol C/ g DW	5102	5689	6458	4374	330	282	318	190	21623	1108	22	19076505
	SD	1507	1685	1488	1057	16	16	70	24	5722	141	9	102885
	CV%	30	30	23	24	5	6	22	12	26	13	40	1
Ulva labeled	nmol C/ g DW	5830	6678	7474	5136	163	131	138	104	25119	536	47	16512437
	SD	1101	1345	1476	1036	32	8	7	27	4956	62	8	1123781
	CV%	19	20	20	20	20	6	5	26	20	12	17	7

Table 4. Stable isotope compositions of RNA and DNA nucleotides in intertidal marine sediment and in a macro alga (*Ulva* sp.) as detected by LC/IRMS in isolated RNA and DNA, hydrolyzed enzymatically by Nuclease P1. Isotopic composition of total organic carbon (TOC) was determined by EA/IRMS. Excess values are the absolute values of produced carbon within the corresponding nucleotides and nucleic acids per 4 h. Stable isotope data and standard deviation (SD) are the average of three replicate samples

		RNA nucleotides				DNA nucleotides					Synthesis rates pmol ^{13}C/h/g DW	
Sample		CMP	AMP	GMP	UMP	dCMP	dAMP	dGMP	dTMP	TOC	RNA	DNA
Sediment	δ^{13}C ‰	−13.6	−8.0	−10.8	−12.4	−14.3	−12.2	−11.7	−19.3	−19.3		
	SD	0.7	0.2	0.3	0.4	0.6	0.2	0.3	0.4	0.1		
Sediment labeled	δ^{13}C ‰	25.6	54.8	51.8	60.0	−8.8	−7.0	−7.5	−12.1	45.1		
	SD	5.0	4.9	5.4	5.2	1.3	1.4	1.3	0.3	8.4		
Ulva	δ^{13}C ‰	−13.5	−9.3	−10.6	−13.3	−18.3	−18.5	−11.2	−17.1	−13.3		
	SD	0.3	0.4	0.1	0.2	0.4	0.2	0.4	0.4	0.1		
Ulva labeled	δ^{13}C ‰	−7.5	64.8	41.8	1.9	−8.7	7.4	−8.1	−13.3	545.7		
	SD	1.1	0.6	0.9	0.3	0.8	0.8	1.2	0.7	11.1		
Sediment labeled	Excess pmol ^{13}C/g DW	34.9	45.4	41.4	35.4	6.2	8.1	5.5	5.1	201800	39	5.8
	SD	6.7	11.9	10.3	7.8	1.8	2.5	1.8	1.3	17500	9	0.5
Ulva labeled	Excess nmol ^{13}C/g DW	0.38	5.42	4.28	0.86	0.017	0.037	0.005	0.005	100567	2732	15.8
	SD	0.04	1.09	0.80	0.19	0.003	0.003	0.002	0.002	8806	524	2.1

sediment and −16.2 ± 0.2 and −11.5 ± 0.2‰ for *Ulva* sp. Both the sediment and *Ulva* sp. RNA $\delta^{13}C$ values are higher than the DNA $\delta^{13}C$ values. There is not a straightforward explanation for this but isotopic discrimination by the biosynthetic pathways must be thought of as a cause for these results. The difference in the $\delta^{13}C$ values of the nucleic acids and the total organic carbon of the sediment is on average approximately 7 ‰, which can be explained by the nucleic acids in the sediment originating from isotopically heavy benthic diatom biomass typically having a $\delta^{13}C$ value between −11 and −18 ‰.[12]

The labeling experiments with the benthic diatom community and *Ulva* sp. indicated a faster ^{13}C labeling of the RNA nucleotides than those of DNA. Hence, we conclude that RNA had a higher turnover than the DNA, which was as expected as RNA is constantly synthesized and broken down. The synthesis rate of DNA in the diatom mat (5.8 ± 0.5 pmol ^{13}C/g DW/h) was of the same order of magnitude as previously reported.[38,39] The reported synthesis rates of RNA in similar marine sediments are somewhat higher than in our samples (39 ± 9 pmol ^{13}C/g DW/h) but it is known that the rate of RNA synthesis depends on the cell cycle and environmental conditions and is therefore variable.[40] The synthesis rate of RNA in *Ulva* sp. was high (2732 ± 524 pmol ^{13}C/h/g DW). Although the RNA nucleotide concentrations were similar, the production of AMP and GMP was a factor of 10 higher than that of CMP and UMP. The asymmetric distribution of the production of RNA nucleotides may be because the pathway that is used for the synthesis of the purine nucleotides (CMP and GMP) differs from that of the pyrimidine nucleotides (AMP and UMP). While the synthesis of the purine nucleotides starts with glucose, that of the pyrimidine nucleotides starts with the combination of carbamoyl phosphate and aspartate.[8] Since ^{13}C is incorporated by photosynthesis with glucose as the initial product, this will be the first cell compound enriched in ^{13}C. Due to the short incubation time (4 h) the ^{13}C labeling of the purine nucleotides will initially be faster than that of the pyrimidines.

This pilot experiment demonstrates that the technique described in this paper has a high potential for tracing DNA and RNA dynamics in organisms and natural ecosystems, and furthermore to determine natural abundance $\delta^{13}C$ values for source studies.[41–43]

CONCLUSIONS

We have developed a method to analyze stable carbon isotope ratios in DNA and RNA nucleotides in a single analytical run by LC/IRMS using mixed-mode chromatography. The solvent-free chromatographic method is not limited to the LC/IRMS application but can also be used to quantify nucleotides by traditional HPLC, for instance in combination with spectrophotometric detection. The method gave reproducible results for the determination of the $\delta^{13}C$ values in nine nucleotides and their concentrations. The big advantage over other methods is that there is no need for extensive incorporation of labeled substrate and excessive incubation times, allowing accurate DNA and RNA synthesis rate determination. This method can be used for the study of metabolic processes in many different research areas. We demonstrated the applicability of the method for the analysis of DNA and RNA nucleotides in marine samples containing micro- and macroalgae at natural ^{13}C abundance level as well as for ^{13}C-labeled material at relatively low enrichments. We show that the method has a high potential for the study of DNA and RNA dynamics in natural environments or organisms. This new method is an attractive addition to the existing protocols for the measurement of carbohydrates and amino acids by LC/IRMS. Although for lipid analysis GC/IRMS remains the preferred method, other structural components, such as proteins, polysaccharides and nucleic acids, can now be studied by LC/IRMS.

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