Application Note: 30055

irm-LC/MS: $\delta^{13}C$ Analysis of RNA

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Key Words

- LC IsoLink™
- HPLC
- Isotope Ratio MS
- Nucleotides

Introduction

Molecular biological methods based on detection, sequencing, and phylogenetic identification of ribosomal RNA (rRNA) and the genes encoding it (rDNA) now allow description of natural microbial communities without isolation and culturing of individual species. However, because of horizontal transfer of "functional" (enzyme-encoding) genes among species, and because newly identified rDNA and rRNA sequences are often only distantly related to those of species that have been studied in pure culture, rRNA phylogeny is not a reliable guide to physiology.

We and others[e.g. [5,9]] have been working to link species identity with carbon source utilization by carbon isotopic characterization of rRNA, either by taking advantage of natural-abundance differences among substrates[2,7] or by addition of labeled compounds[6]. The major hurdle has been the isolation of sufficient rRNA, free of other carbon compounds, for reliable isotopic determinations by elemental analyzer isotope ratio MS. Attempts to derivatize either the nucleobase or ribose moieties of hydrolyzed RNA for *irm*-GC/MS were hampered by low reaction efficiencies and substantial isotope effects (A. Miltner, M. Gehre, V. Brüchert, M. Kästner, B. MacGregor; unpublished).

With the development of the Thermo Scientific LC IsoLink interface[4], compounds can now be separated by HPLC and analyzed online for their individual $^{13}C/^{12}C$ ratios by isotope ratio MS. Here we report progress towards a method for HPLC separation of hydrolyzed RNA and determination of the carbon isotopic composition of the monomers.

irm-LC/MS Technology

The LC IsoLink is the first high sensitivity interface connecting HPLC with Isotope Ratio MS for the reproducible and accurate on-line determination of $^{13}C/^{12}C$ isotope ratios. All organic compounds eluting from an HPLC column are analyzed while maintaining the chromatographic resolution.

In the LC IsoLink, the sample is oxidized to CO_2 within the aqueous solvent eluting from the HPLC. The CO_2 is separated from the liquid phase and fed into the IRMS ion source. The oxidation reagent consists of two solutions, the oxidizing agent and phosphoric acid, both of which are pumped separately and added to the mobile LC phase.

Within this mixture, all organic compounds eluting from the HPLC column are oxidized quantitatively into CO_2 when passing through a heated reactor. In a downstream separation unit, the CO_2 is removed from the liquid phase and entrained into a stream of He. The individual CO_2 peaks in He (which correspond one-to-one with the peaks of the individual compounds) are subsequently dried in an on-line unit (Nafion®) and then admitted to the Isotope Ratio MS via an open split interface.

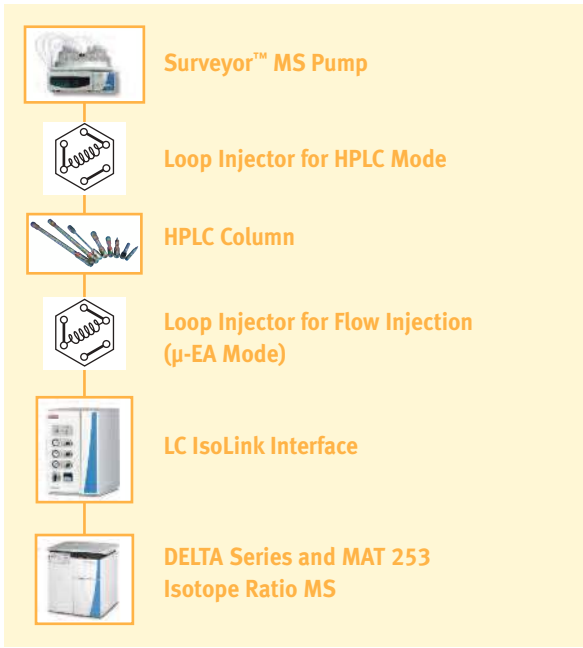

Figure 2: Scheme of the Thermo Scientific *irm*-LC/MS system with the LC IsoLink.

Experimental Section

Sample preparation was performed by Barbara MacGregor and Adam Friedman from University of North Carolina, Chapel Hill, USA. *irm*-LC/MS analysis was carried out in the applicaton laboratory of Thermo Fisher Scientific, Bremen, Germany.

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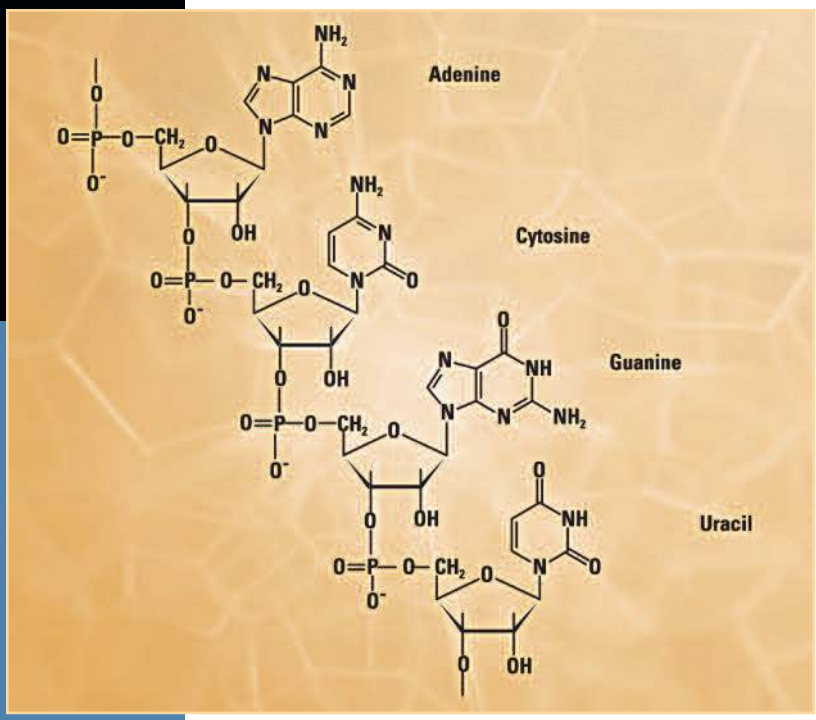

Cell Growth and RNA Isolation

All solutions were prepared with diethylpyrocarbonate (DEPC)-treated deionized water. *Escherichia coli* DSM 1103 was grown on LB medium, or on M9 minimal medium with either glucose or acetate as the sole carbon source. Cells were lysed with a FastPrep Instrument (Bio 101 Inc., Buena Vista, California), with two 20 sec shaking cycles at speed 6, and RNA was isolated by phenol-chloroform extraction[8,10] and isopropanol precipitation. Further purifications were performed, as indicated, by CTAB treatment[3], digestion with RNase-free DNase I (Ambion Inc., Austin, Texas), and/or passage through MinElute columns (QIAGEN Inc., Valencia, California), reagents and growth media were from Thermo Fisher Scientific.

HPLC Separation

RNA samples were digested to mononucleotides by incubation in 0.2 N NaOH for 15 min at 50 °C. Nucleotides were separated by chromatography on a HyPURITY AQUASTAR™ column (particle size 5 µM; Thermo Fisher Scientific) with 0.1 M potassium phosphate buffer (pH 6.0). Simple HPLC runs were done for 80 min on a 150 mm x 4.6 mm column at 25 °C, with a flow rate of 0.5 mL min^{-1}. The HPLC instrument has a BioRad 2800 gradient pump with a Shimadzu SPD10-AV detector. Detection was at 254 nm. Nucleotide standards were purchased from Sigma-Aldrich Co.

irm-LC/MS Analysis

The LC IsoLink interface can be used in either of two operational modes, one for compound specific isotope analysis (*irm*-LC/MS mode) and one for bulk stable isotope analysis (µ-EA mode).

The interface was coupled with the Thermo Scientific Surveyor™ HPLC system using a HyPURITY AQUASTAR column (150 mm x 2.1 mm, particle size 5 µM; Thermo Fisher Scientific) with 0.1 M potassium phosphate buffer (pH 6.0). The separations for *irm*-LC/MS were done for 42 min at 25 °C with a flow rate of 0.5 mL min^{-1}.

The oxidation is obtained by adding oxidation reagents to the mobile phase. The oxidation reagents consist of the oxidant, i.e. sodium peroxodisulfate, and phosphoric acid.

HPLC-grade water is used for reagent preparation. The solutions are degassed under vacuum in an ultrasonic bath. The reagents are pumped separately allowing different flow rates. The reaction is carried out in a reactor maintained at 99.9 °C. Table 1 shows the detailed analytical conditions that were applied for HPLC and the *irm*-LC/MS interface.

HPLC Parameters

Pump:	Thermo Scientific Surveyor™ MS Pump
Mobile Phase:	100 mM KH_2PO_4, pH 5 degassed with helium
HPLC Column:	Thermo Scientific HyPURITY AQUASTAR™, 150 mm x 2.1 mm, particle size: 5 µm spherical
Temperature:	ambient
Flow:	400 µl/min
Loop:	20 µl

Interface Parameters

Reagent Pump 1:	1.05 M $Na_2S_2O_8$ Flow: 70 µl/min
Reagent Pump 2:	1.3 M H_3PO_4 (density: 1.88 g/cm^3) Flow: 70 µl/min
Reactor Temperature:	99.9 °C
Sample Loop 2:	5 µl

Table 1: HPLC and LC IsoLink parameters.

Results and Discussion

HPLC Separation of RNA Nucleotides

A protocol for HPLC separation of deoxyribonucleotides was adapted for RNA (Figure 1). Critical factors were 1) increasing the run time from 20 to 80 minutes (for the 4.6 mm diameter column), and 2) rinsing the column frequently with a water/methanol/water series (not shown).

Homopolymers of ribonucleotides, or total *Escherichia coli* RNA, were hydrolyzed under basic conditions before chromatography. Base hydrolysis of RNA yields 2´,3´-cyclic monophosphates, which resolve to a mixture of 2´- and 3´- monophosphates. The polypyrimidine polyU yielded a single UV-absorbent peak (Figure 1A), while the polypurines polyA and polyG yielded two peaks each (Figure 1A, B). (PolyC was not available).

Hydrolysis of polypyrimidines may favor one monophosphate isomer over the other, or the HPLC mobilities of the monophosphates may differ only for purines; the HPLC mobility of various 2´- and 3´-monophosphates will be tested to address this question. Importantly, all hydrolysis products of an *E. coli* RNA sample could be identified (Figure 1C), assuming that the first peak, which eluted faster than the standards tested, is C.

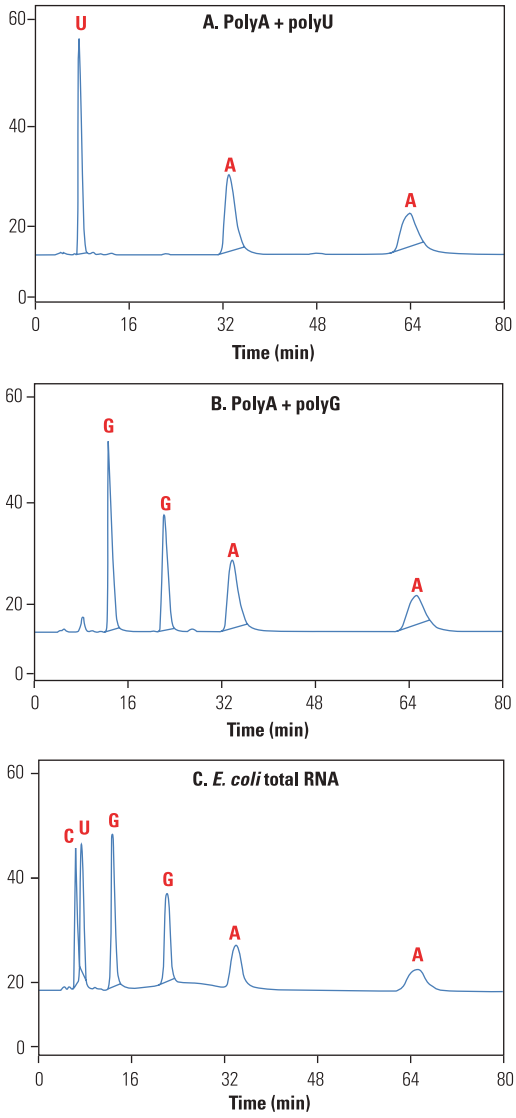

Figure 1: HPLC separation of NaOH-hydrolyzed polynucleotides and RNA. Approximately 4 µg of RNA were digested to mononucleotides by 15 min incubation at 50 °C in 0.2 N NaOH, and separated by HPLC as described in the Experimental section.

Carbon Isotopic Determination of RNA Nucleotides by *irm*-LC/MS

The HPLC protocol described above was then used to separate hydrolyzed polynucleotides for combustion by the LC IsoLink interface. As expected, a single peak was seen for hydrolyzed polyU (Figure 2A), and pairs of peaks with identical $\delta^{13}C$ values for polyG and polyA (Figure 2B, C). The small initial peaks in the polyG and polyA runs and the very shallow peak in the polyG run have not yet been identified. When determined, these had different ^{13}C values than the presumed nucleotide peaks (e.g. Figure 2C), suggesting that they do not derive from sample RNA. Similar UV-detectable peaks seen in the course of HPLC method development (not shown) were attributed to sample carryover, since they could be eliminated or reduced by rinsing the column with a water / methanol / water series between runs. Alternatively, there may be carbon compounds present that are not detectable by UV absorbance.

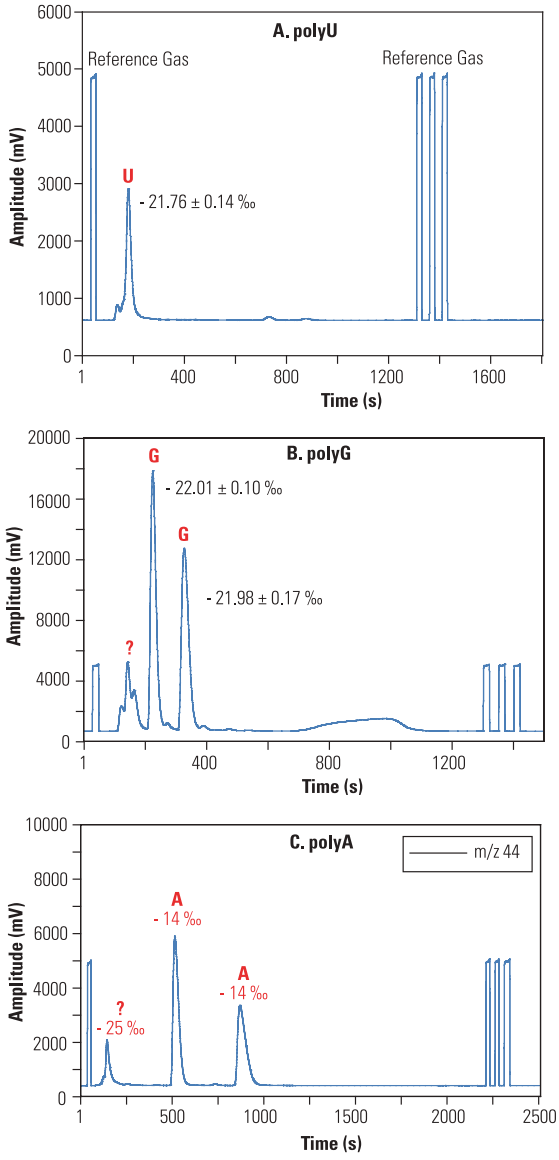

Figure 2: *irm*-LC/MS analysis of NaOH-hydrolyzed polynucleotides. RNA hydrolysis and HPLC separations were done as for the experiments in Figure 1, except that a 2.1 mm diameter column was used. Numbers next to peaks are $\delta^{13}C$ (PDB) values.

Carbon Isotopic Determination of Bacterial RNA by *irm*-LC/MS

Isotope ratio analysis of RNA isolated from *E. coli* grown on different carbon substrates was investigated next. Earlier work with EA isotope ratio MS indicated that the carbon isotopic composition of RNA closely reflects that of the growth substrate[7]. That appears to be the case for the two peaks identified as adenine in the *irm*-LC/MS separations as well (Figure 3).

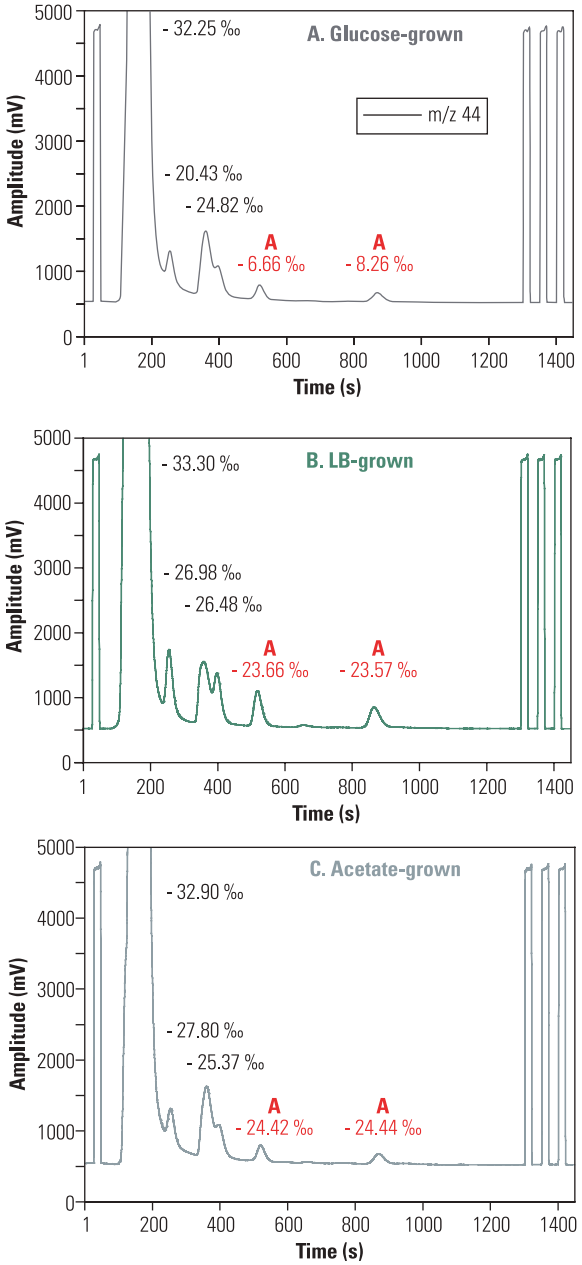

Figure 3: *irm*-LC/MS analysis of NaOH-hydrolyzed *E. coli* RNA. RNA was extracted from overnight cultures of *E. coli* grown on LB medium, or on M9 minimal salts with glucose or acetate as sole carbon source[6]; denatured in 0.2 N NaOH; separated by HPLC on a 2.1 mm diameter HyPURITY AQUASTAR column; and introduced to the IRMS via the LC IsoLink interface. $\delta^{13}C$ values are the average of three determinations; errors were < 1 ‰ and are shown in Figure 4.

RNA-derived adenine from cells grown on glucose (apparently produced from a C4 plant) was isotopically heavier than RNA from cells grown on either LB or acetate. However, all three samples also yielded several early-eluting, isotopically light peaks.

Comparison of the five main peaks common to the three samples suggests the C, U, and G peaks may be obscured by contaminants (Figure 4).

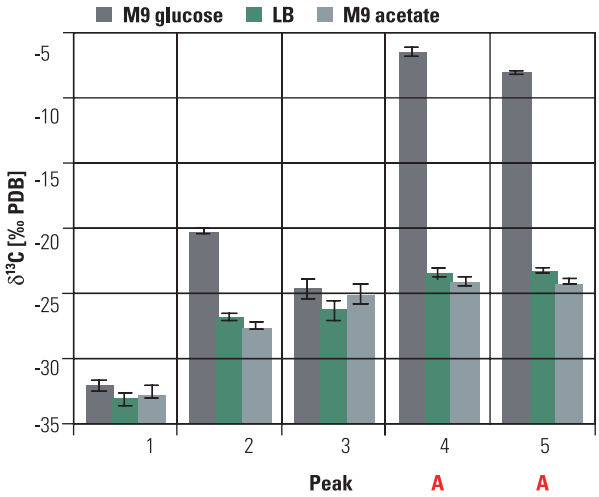

Figure 4: Carbon isotopic composition of individual peaks from NaOH-hydrolyzed *E. coli* RNA. Peaks are numbered from first- to last-eluting (see Figure 3).

While peaks 1-3 for the glucose-grown cells are isotopically lighter than the presumptive adenine peaks 4 and 5, peak 2 from glucose-grown cells is significantly more en-riched in ^{13}C than it is for the LB- or acetate-grown cells; that is, closer to the value expected for glucose-derived carbon. Non-RNA carbon, whether from the samples or from the HPLC and combustion procedures, may be co-eluting with ribonucleotides. The source of this carbon will be investigated by testing different RNA purification and column washing procedures, as well as by blank runs.

Conclusions

The preliminary experiments have raised questions that should be addressed before beginning work with environmental samples. First, why are there two main HPLC peaks for hydrolyzed polyA and polyG, but only one for polyU and likely polyC (Figs. 1, 2)? Second, the apparent contaminant peaks in the RNA samples (Figure 3) must be removed, or at least identified. When *E. coli* RNA was analyzed by total combustion, the isotopically heavy values obtained from glucose-grown cells gave little evidence for contamination by large amounts of isotopically light carbon[7]. Nor were large extraneous peaks detected by UV absorbance following simple HPLC separation of hydrolyzed RNA (Figure 1C). This suggests that isotopically light carbon lacking UV absorbance may be introduced during the RNA hydrolysis or HPLC procedures.

irm-LC/MS analysis of rRNA will be applied in ongoing projects with Dr. H.T.S. Boschker (Netherlands Institute of Ecology, Yerseke, The Netherlands) concerning the role of prokaryotes vs. eukaryotes in carbon cycling in the Scheldt River (Netherlands) estuary[6], and with Dr. Nicole Dubilier (Max Planck Institute for Marine Microbiology, Bremen, Germany) concerning the contribution of methanotrophic vs. autotrophic symbionts to the carbon nutrition of hydrothermal vent mussels[1,2]. In both cases, magnetic bead capture hybridization[7] was used to isolate small-subunit ribosomal RNA from different phylogenetic groups, the carbon isotopic composition of which was measured by EA isotope ratio MS. It has been difficult to completely rule out contamination by polysaccharides and humic substances in microgram-scale samples. *irm*-LC/MS should greatly reduce these concerns. We hope that its increased sensitivity will also allow us to look at a finer level of phylogenetic detail; for example, to identify the carbon sources incorporated by specific groups of sulfate-reducing bacteria in the estuarine sediments. The Thermo Scientific LC IsoLink interface has the potential to be useful in determining trophic structure and carbon flow in microbial communities in a wide range of marine and terrestrial ecosystems.

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Barbara MacGregor is a microbial ecologist, interested in using ribosomal RNA as a tool to follow carbon flow in natural populations of bacteria and archaea. She obtained a B.S. in Biology and an M.S. in Marine Microbiology at the University of Massachusetts - Boston, a Ph.D. in Bacteriology at the University of Wisconsin - Madison, and did postdoctoral research with Dr. David Stahl at Northwestern University (Evanston, Illinois) and Dr. Rudolf Amann at the Max Planck Institute for Marine Microbiology (Bremen, Germany). She is currently a Research Assistant Professor in the Department of Marine Sciences at the University of North Carolina - Chapel Hill.

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