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RESEARCH ARTICLE

Stable Isotope Labeling for Improved Comparative Analysis of RNA Digests by Mass Spectrometry

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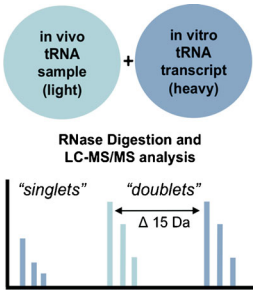

Abstract. Even with the advent of high throughput methods to detect modified ribonucleic acids (RNAs), mass spectrometry remains a reliable method to detect, characterize, and place post-transcriptional modifications within an RNA sequence. Here we have developed a stable isotope labeling comparative analysis of RNA digests (SIL-CARD) approach, which improves upon the original $^{18}O/^{16}O$ labeling CARD method. Like the original, SIL-CARD allows sequence or modification information from a previously uncharacterized in vivo RNA sample to be obtained by direct comparison with a reference RNA, the sequence of which is known. This reference is in vitro transcribed using a $^{13}C/^{15}N$ isotopically enriched nucleoside triphosphate (NTP). The two RNAs are digested with an endonuclease, the specificity of which matches the labeled NTP used for transcription. As proof of concept, several transfer RNAs (tRNAs) were characterized by SIL-CARD, where labeled guanosine triphosphate was used for the reference in vitro transcription. RNase T1 digestion products from the in vitro transcript will be 15 Da higher in mass than the same digestion products from the in vivo tRNA that are unmodified, leading to a doublet in the mass spectrum. Singlets, rather than doublets, arise if a sequence variation or a post-transcriptional modification is present that results in a relative mass shift different from 15 Da. Moreover, the use of the in vitro synthesized tRNA transcript allows for quantitative measurement of RNA abundance. Overall, SIL-CARD simplifies data analysis and enhances quantitative RNA modification mapping by mass spectrometry.

Keywords: Stable isotope labeling, Modified nucleosides, RNA sequencing, tRNA, Tandem mass spectrometry, LC-MS/MS

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Introduction

Ribonucleic acid (RNA) is a central molecule in the flow of genomic information from DNA to proteins. Post-transcriptional modifications in RNA are more prevalent and structurally distinct than the ones found in DNA [1]. Transfer RNA (tRNA) harbors the largest set of these modifications, of which more than 100 have been discovered in the three domains of life [2, 3]. Modifications often involve additions of a chemical group (i.e., methylations), atom exchange (S to O exchange), or base exchange (guanosine to queuosine), where an analogue replaces the canonical nucleobase [4–7]. Recently, it has been found that RNA modifications are dynamic and responsive to environmental stress, thus providing another layer of gene expression regulation [8–11].

Mass spectrometry (MS) is one of the most reliable methods of directly characterizing modified nucleosides, as chemical modifications increase the mass of a canonical nucleoside [12–14]. Collision induced dissociation tandem mass spectrometry (CID MS/MS) can be used to reveal the location of a modified nucleoside within the sequence of the RNA being studied [15]. RNA modification mapping or RNase mapping is the most commonly used MS strategy to identify and locate multiple modifications within one or more RNA sequences. In this bottom-up approach, the RNA is digested with RNA-specific enzymes (RNases) producing mixtures of oligoribonucleotides that can be directly analyzed by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) or characterized by on-line high performance liquid chromatography (HPLC) electrospray ionization mass spectrometry (ESI-MS) [16–19].

While newer high-throughput approaches have demonstrated successes in global characterization of specific modifications or modification motifs [20], RNA modification mapping

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by mass spectrometry has been an enabling approach for the characterization of modified ribosomal RNAs (rRNAs) and tRNAs [14, 18, 21–23]. However, richer data sets that enable more biological information should contain both qualitative information (e.g., modification identity and sequence location) along with quantitative information (i.e., is a particular modification present in every copy of a single RNA?). Thus, more recent developments have focused on incorporating quantitative techniques by isotopic labeling into the RNA modification mapping strategy [24–27].

The first demonstration of a metabolic labeling approach was presented by Waghmare and Dickman [25]. They showed that by growing cells in enriched media, one can generate an appropriately labeled standard, which can be used for both qualitative and quantitative applications. RNAs isolated from unlabeled and labeled cells are processed as in the usual RNA modification mapping protocol. These authors found that by combining light (^{14}N) and heavy (^{15}N) rRNA isolated from *Escherichia coli* an accurate assignment of nucleotide composition is achieved, fragments bearing modifications can be assigned with confidence, and compositional isomers that often co-elute can be discriminated by comparing the light and heavy isotope patterns without the need for a high mass accuracy mass spectrometer. Adapting this method, Popova and Williamson used mature ^{15}N-labeled rRNA from *E. coli* as an internal standard to understand the mechanism of rRNA biogenesis by measuring site-specific changes in modification stoichiometry for these rRNAs [26]. They extended this approach by supplementing the growth medium with CD_3-methionine and 5,6-D-uracil to enable selective quantification of methylations and pseudouridine, respectively.

An alternative strategy for quantitative RNA modification mapping was demonstrated by Taoka et al. who used in vitro transcribed RNAs generated from isotopically labeled precursors (i.e., $^{13}C_{10}$-guanosine triphosphate, GTP) [27]. The two key differences between this in vitro strategy and the in vivo strategies described above arise in the ability to perform absolute quantification with an in vitro standard and the use of an internal standard lacking any modifications. These researchers have used this stable isotope-labeled ribonucleic acid as an internal standard (SILNAS) approach to quantitatively characterize individual RNAs of limited modification diversity from *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* [27, 28].

All of the quantitative RNA modification mapping approaches described above are based on the mass spectrometry detection and characterization of characteristic doublets or singlets. A similar strategy – limited to qualitative applications – was developed by our group for the characterization of tRNA samples. The method, called comparative analysis of RNA digests (CARD) by stable isotope labeling [29, 30], requires a reference RNA of known sequence and modification profile, and a candidate RNA, which should share sequence similarity with the reference, yet its modification profile is unknown. The digestion products are differentially labeled by light ($H_2{}^{16}O$) and heavy water ($H_2{}^{18}O$) during RNase digestion, then combined and analyzed by LC-MS/MS. If the digestion product sequences are identical, those digestion products will have the same mass and appear in the mass spectrum as a characteristic doublet, separated by 2 Da due to the $^{18}O/^{16}O$ mass difference. Singlets will arise if the RNA sequences are different between the reference and sample and when the sample is post-transcriptionally modified differently than the reference. This method is suitable for single or total tRNA comparison, gene deletion mutants, and modification placement for unknown organisms.

Despite the utility of CARD for rapid evaluation of total tRNA modification profiles, it cannot be used to obtain quantitative information as spectral congestion due to the naturally occurring isotopes of carbon (^{13}C) and nitrogen (^{15}N) make data analysis quite challenging [24]. Furthermore, it is increasingly difficult to distinguish doublets separated by only 2 Da as the charge state of the RNase digestion products increase unless high mass accuracy mass analyzers are used. This small label mass shift hampers the ability to accurately characterize and quantify RNA in complex mixtures, where the size of the digestion products can vary from 6- to 20-mers. We recently addressed some of these limitations by the metabolic labeling approach wherein cells for one of the two required samples were grown in a ^{12}C enriched/^{13}C depleted medium. This metabolic approach significantly reduced isotopic overlaps, eased spectral congestion, and simplified analysis of singlets and doublets [31].

However, the CARD approach still utilized enzymatic labeling through RNase digestion to create the two samples for analysis, which is limiting due to the 2 Da separation that is achieved by using heavy water. Here we seek to overcome this method limitation by adapting the in vitro internal standard approach demonstrated by Taoka et al. [27] to the overall CARD strategy for tRNA characterization. The SIL-CARD approach is more widely applicable for tRNA analysis as one only needs to know the genomic information for the tRNA(s) of interest to generate the isotopically labeled transcript(s) as a reference. Doublets are easily characterized by the mass shift due to the ^{13}C and ^{15}N enrichment in isotopically labeled precursor, which can be tailored to the RNase of interest. With SIL-CARD, the advantages of previous quantitative approaches to RNA modification mapping can now be brought to bear on another class of important modified RNAs.

Experimental

Materials

E. coli tRNA Tyr II was purchased from Sigma Aldrich (St. Louis, MO, USA). Deoxynucleotide triphosphate mix, *PfuI* DNA polymerase, and 10X PCR buffer were used as received from Promega (Madison, WI, USA). Triethylamine (TEA), 1,1,1,3,3,3-hexafluoroisopropanol (HFIP), ammonium acetate, ethanol, and MEGAClear Transcription kit were obtained from Thermo Fischer Scientific (Waltham, MA, USA). Qiagen QIAquick (Hilden, Germany) and EpiCenter AmpliScribe

(Madison, WI, USA) kits were used. The ^{13}C, ^{15}N labeled GTP (98% atom) was purchased from Sigma Aldrich). HPLC-grade methanol and acetonitrile were obtained from Honeywell Burdick and Jackson, Inc. (Muskegon, MI, USA). UltraPure agarose was purchased from Invitrogen Corporation (Carlsbad, CA, USA). RNase T1 and bacterial alkaline phosphatase were obtained from Worthington (Lakewood, N,USA). Sep-Pak C18 cartridges were obtained from Waters (Milford, MA, USA). Nanopure water (18 MOhms) from a Barnstead (Dubuque, IA, USA) nanopure system was used throughout.

Amplification of E. coli tDNA Tyr II by Polymerase Chain Reaction (PCR)

The gblocks gene fragments for *E. coli* tDNA tyrosine II, forward primer (which contains the T7 RNA polymerase promoter sequence) and reverse primer (Table 1) were obtained from Integrated DNA Technologies, Inc. (Coralville, IA, USA). The PCR master mix was prepared following the vendor protocol. For the negative control, the same master mix was prepared without the gene fragment. Amplification was as follows: initial denaturation for 2 min at 94 °C, denaturation, annealing, and extension at 92 °C, 55 °C, 72 °C for 30 s each, and the cycle was repeated 28 times. Final extension was held for 5 min at 72 °C. The PCR products were purified by a QIAquick cleanup kit and analyzed on a 1.5% agarose gel with standard DNA marker. The amplified tDNA was used as the template for subsequent T7 RNA in vitro transcription.

In Vitro Transcription of Light (Regular) and Heavy (Isotopically Enriched) E. coli tRNA Tyr II

The transcription reactions were assembled from AmpliScribe high yield transcription kit: tDNA template (PCR product), 10× reaction buffer, dithiothreitol (DTT), RNase inhibitor, inorganic phosphatase, T7 RNA polymerase, ATP, CTP, UTP, and GTP in a total volume of 50 μL maintained by addition of sterile water. For isotopically enriched transcripts, GTP was replaced by ^{13}C, ^{15}N labeled GTP (98% atom, Sigma Aldrich). After 1 h incubation at 37 °C, DNase I and EDTA were added to quench the reaction. The transcripts were purified (MEGAClear transcription kit) following vendor instructions. The RNA was quantified using an Implen Pearl NanoPhotometer (Implen GmbH, Munich, Germany).

RNase T1 Digestion

All RNA samples were digested using RNase T1, which was purified as previously described [24]. CARD analyses involved combining 1 μg of isotopically labeled transcript with 1 μg of *E. coli* tRNA Tyr II standard prior to enzymatic digestion. The samples were digested with 50 U RNase T1 per μg RNA for 2 h at 37 °C, vacuum dried, and stored at –20 °C until further analysis. Samples were resuspended with 10 μL of mobile phase A prior to LC-MS/MS analysis.

Liquid Chromatography and Tandem Mass Spectrometry

The RNase digestion products were separated on an Xbridge-MS C18 1.0 × 150 mm, 3.5 μm particle size and 150 Å pore size (Waters, Milford, MA, USA) at a flow rate of 30 μL min^{-1}. Mobile phase A (MPA) consists of 8 mM TEA/200 mM HFIP, pH 7, and mobile phase B is 50% MPA and methanol. The LC gradient initiated at 10% B, then increased linearly to 60% B for 32 min, followed by 95% B for 5 min before a minimum 20 min re-equilibration period at 10% B.

All LC-MS/MS analyses were performed using a MicroAS autosampler, Surveyor MS pump HPLC system, and Thermo LTQ-XL (Thermo Scientific, Waltham, MA, USA) linear ion trap mass spectrometer with an ESI source. The capillary temperature was set at 275 °C, spray voltage of 4 kV, and 35, 14, and 10 arbitrary flow units of sheath, auxiliary, and sweep gas, respectively. The mass spectra were recorded in negative polarity and profile data type. Scan event 1 was set at zoom scan with a mass range of 500–2000 *m/z*. Sequence information of the four most abundant digestion products were obtained in scan events 2–5 by data-dependent CID. Data acquisition was through Thermo Xcalibur software.

Calibration Curve and Data Analysis

A calibration curve was generated by analyzing mixtures of heavy (isotopically enriched transcripts) and light (regular transcripts). The heavy and light transcripts were combined in ratios of 10:1 to 1:10 ([heavy]/[light]). Each mixture was prepared and analyzed in triplicate. The ion abundance of RNase T1 digestion products were measured using extracted ion chromatograms (XIC) of the major isotope. The ion abundance ratio, averaged from the extracted ion chromatogram, was calculated by the use of Equation 1,

$$\frac{I^*_{heavy}}{I_{light}} = \frac{I^*_{A+15}}{I_A} \quad (1)$$

where I_A represents the monoisotopic peak abundance of the unlabeled digestion product, and I_{A+15} represents the monoisotopic peak abundance of the labeled digestion product. The characteristic mass shift of 15 Da is due to the $^{13}C_{10}$ and $^{15}N_5$ in the GTP precursor. All ratios were corrected for the 98% isotopic purity of GTP. The arithmetic mean and standard

Table 1. The Gene Sequences of *E. coli* tRNA Tyr II Forward Primer (with the T7 RNA Polymerase Promoter Region) and Reverse Primer

tDNA	GGTGGGGTTCCCGAGCGGCCAAAGGGAGCAGACTGTAAATCTGCCGTCATCGAC TTCGAAGGTTCGAATCCTTCCCCCACCACCATTAAGCTTTCCGGAT
Forward primer	GGATCCTAATACGACTCACTATAGGGGTGGGGTTCCCGAGCGGCCAAA
Reverse primer	TTTTGAGCTTTGGTGGTGGGGGAA

deviation were calculated for each ratio. A calibration curve representing the mean values of ion abundances and concentration ratios were constructed by a linear least squares fit.

The *E. coli* tRNA Tyr II gene sequence was obtained from the Genomic tRNA Database (http://gtrnadb.ucsc.edu/) [32]. The *E. coli* Tyr II sequence annotated with post-transcriptional modifications was obtained from Modomics (http://modomics.genesilico.pl/) [33]. The Mongo-Oligo Calculator (http://mods.rna.albany.edu/masspec/Mongo-Oligo) was used to calculate the theoretical molecular mass of the RNase T1 digestion products from all RNA sequences, and these masses were used to identify the appropriate XIC channels to monitor during quantitative analyses.

Results and Discussion

The advantages of a transcript-based in vitro synthesized internal standard or reference for the CARD approach were recognized within the initial report by Taoka et al. [27]. Our initial goal, described here, was to examine the utility of such transcripts for the quantitative characterization of individual tRNAs. However, the method itself should be readily extended to more complex mixtures of tRNAs although that has not yet been explored. A schematic illustration of our SIL-CARD workflow is shown in Figure 1. Isotopically labeled GTP precursor is also used here, as RNase T1 digestion will ensure that each heavy digestion product (except the 3′-terminus of the tRNA) will have the characteristic 15 Da mass shift.

Identification and Characterization of In Vitro Transcribed E. coli tRNA Tyr II

To verify that isotopically labeled transcripts would co-elute and provide sufficient *m/z* difference to be used in the CARD method, light (regular) and heavy (isotopically enriched) transcripts of tRNA tyrosine II (GUA) patterned from *E. coli* were synthesized. Equal amounts of the light and heavy transcripts were combined and digested with RNase T1 to generate a series of oligonucleotides, which were then separated by ion pairing liquid chromatography and detected by ESI-MS/MS. A representative example of the data obtained is shown in Figure 2. Here, the expected digestion product UUCGp is detected from both the light (*m/z* 639.1, –2 charge state) and heavy (*m/z* 646.6, –2 charge state) transcripts. The mass shift of 15 Da is as expected based on the single labeled guanosine. These digestion products co-elute, showing no retention time effects due to the labeled guanosine as expected based on previous reports (Figure 2a) [27].

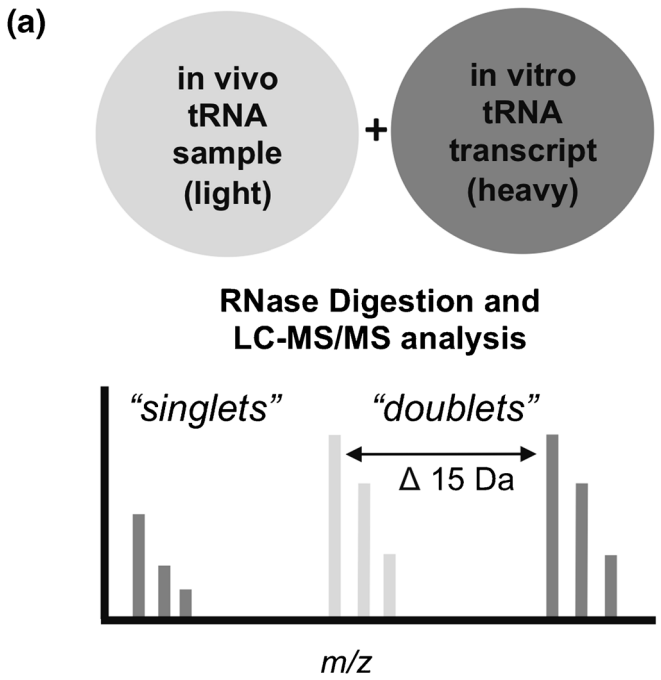
Due to the reproducible nature of CID of RNase T1 digestion products, the y-type fragment ion series should contain the isotopic label for the heavy transcript. In other words, typical c-type fragment ion series for the two samples should be identical, but the y-type fragments will differ by the +15 Da mass shift due to the labeled guanosine on the 3′-terminus of each RNase T1 digestion product. As seen in Figure 2b and c, indeed c-type fragment ions are identical for these two digestion products but the y-type fragment ions contain the expected mass shift. These data confirm the localization of the isotopic label to only the guanosine residue. A similar analysis was performed on all other expected RNase T1 digestion products from both the light and heavy transcripts to confirm their utility in the SIL-CARD approach (data not shown).

Use of Heavy Transcript as CARD Reference

As an initial demonstration that in vitro transcripts can be used for CARD, the isotopically labeled transcript of *E. coli* tRNA Tyr II and an *E. coli* tRNA Tyr II standard were combined in equal amounts and analyzed by LC-MS/MS. Based on the known modification profile of *E. coli* tRNA Tyr II, three CARD doublets should be found in the mass spectral data: CCAAAGp, UCACAGp, and ACUUCGp. RNase T1 digestion products less than 4-mer were not considered. Because the transcript is unmodified and the standard used here is of known modification profile, there are three singlets from *E. coli* tRNA Tyr II anticipated in the experimental data: [m^5U][Ψ]CGp, [s^4U][s^4U]CCCGp, and ACU[Q]UA[ms^2i^6A]A[Ψ]CUGp. Two of these RNase digestion products should be detected as unmodified equivalent singlets from the in vitro reference: UUCG*p and UUCCCG*p. The transcript equivalent of ACU[Q]UA[ms^2i^6A]A[Ψ]CUGp should not be detected, as the modified nucleoside queuosine is generated by post-transcriptional base-exchange at G34 in *E. coli* tRNA Tyr II. Thus, the transcript yields two unmodified digestion products, ACUG*p and UAAAUCUG*p.

The first step in standard CARD analysis is to examine the data for doublets. Figure 3 contains the predicted doublets, which differ in mass only at the terminal guanosine residue. Figure 4 shows the detection of the singlets predicted from a CARD analysis performed in this manner. Figure 4a reveals the expected *m/z* values for UUCG*p and its modified equivalent. Similarly, Figure 4b reveals the expected *m/z* value for UUCCCG*p and its modified equivalent. In both cases, the post-transcriptional modifications lead to elution time differences between the reference and sample digestion products. Figure 4c confirms that the unmodified transcript yields two digestion products, which are the sequence equivalent of the modified product ACU[Q]UA[ms^2i^6A]A[Ψ]CUGp.

These data demonstrate that in vitro transcripts can be used as the reference for CARD characterization of tRNAs. Although prior versions of the CARD approach used cellular tRNAs containing known modification profiles as the reference, the initial data analysis is similar when using transcripts. In those earlier iterations of the method, the data is first examined to identify all doublets, which are used to confirm sequence and modification equivalency between the reference and the sample. After that step, the data is then examined for the missing doublets (i.e., singlets). While prior versions of CARD require a reverse labeling step to differentiate singlet source (either reference or sample) [29], the use of an unmodified transcript eliminates the need for any reverse labeling as



(b)

In vitro transcript

5'–pppGGUGGGGUUCCCGAGC
GGCCAAAGGGAGCAGACUGUA
AAUCUGCCGUCACAGACUUCG
AAGGUUCGAAUCCUUCCCCCA
CCACCA–3'

E. coli Tyr II :

5'- pGGUGGGG[s^{4}U[s^{4}U]CCCGA
GCGGCCAAAGGGAGCAGACU
[Q]UA[ms^{2}i^{6}A]AUCUGCCGUCACA
GACUUCGAAGG[m^{5}U[Ψ]CGAAU
CCUUCCCCCACCACCA -3'

Figure 1. **(a)** Schematic outline of stable isotope labeling- comparative analysis of RNA digest (SIL-CARD). An in vitro transcribed RNA, synthesized with ^{13}C and ^{15}N enriched GTP, is the reference for the RNA sample. When RNase T1 digestion products of the sample are unmodified, the mass spectrum will reveal a doublet, separated by 15 Da, from the sample and reference. Singlets, which contain post-transcriptional modifications, can be characterized by MS/MS. **(b)** Sequences for the *E. coli* tRNA Tyr II transcript (reference) and *E. coli* tRNA Tyr II RNA (sample). The sequences can be digested theoretically (i.e. RNase T1) to generate a predicted list of singlets and doublets

the *m/z* values for all transcript digestion products will be known before analysis and only these digestion products would contain the characteristic isotopically labeled guanosine at the 3′-terminus of the digestion product.

Quantitative Utility of CARD References

After characterizing the advantages of isotopically labeled transcripts for use as the reference in a CARD approach, we next sought to confirm that these transcripts – after enzymatic digestion and LC-MS – could also serve as internal standards to obtain quantitative information from a CARD experiment. To establish quantification of RNase T1 digestion products, a linear correlation between sample:reference ratios and the detected ion abundance ratios should exist. The unlabeled and labeled *E. coli* tRNA Tyr II transcripts were combined in concentration ratios ranging from 10:1 to 1:10 [labeled/unlabeled], digested with RNase T1, and analyzed by LC-MS.

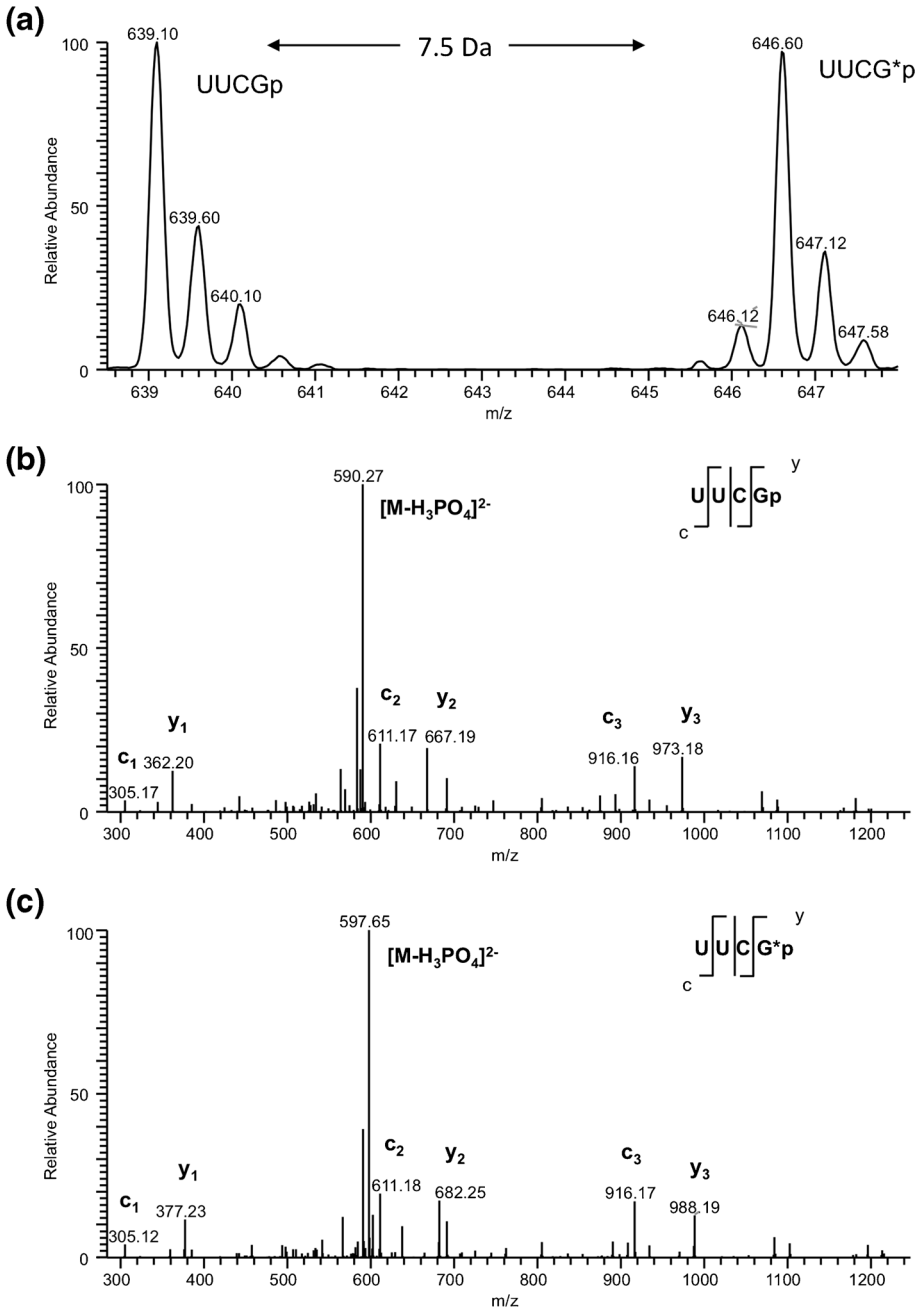


Figure 2. Mass spectral data for the *E. coli* tRNA Tyr II RNase T1 digestion product UUCGp. **(a)** The doubly charged digestion products from both sample and reference are detected with the expected mass difference of 15 Da. MS/MS of the **(b)** sample and **(c)** reference digestion products. The 15 Da increase in the y-type fragment ions of the reference is due to the ^{13}C and ^{15}N labeled GTP. *Denotes isotopically labeled oligonucleotide

Surprisingly, a nonlinear response was noted that was independent of the LC and ESI-MS conditions used during analysis (data not shown). However, when the 3′ phosphate group from each RNase T1 digestion product was removed by bacterial alkaline phosphatase prior to LC-MS analysis, a more well-behaved linear relationship between concentration ratios and ion abundance ratios was found (Figure 5). The reason for nonlinear responses for 3′-phosphate containing digestion products is not yet known, but additional studies are underway to better characterize those anomalous results.

Representative mass spectra of different mixtures of the doubly charged digestion product UUCCCG are shown in Figure 5a. As seen in these examples, the 15 Da mass difference more easily enables quantification compared with prior CARD approaches. Ion abundance ratios were calculated using Equation 1 and are found in Table 2. The RSD for these ratios were within 10% throughout. From these data, a representative calibration curve was generated as shown in Figure 5b. These data are well-behaved when analyzed by linear least squares, yielding a slope close to 1.0 with only a

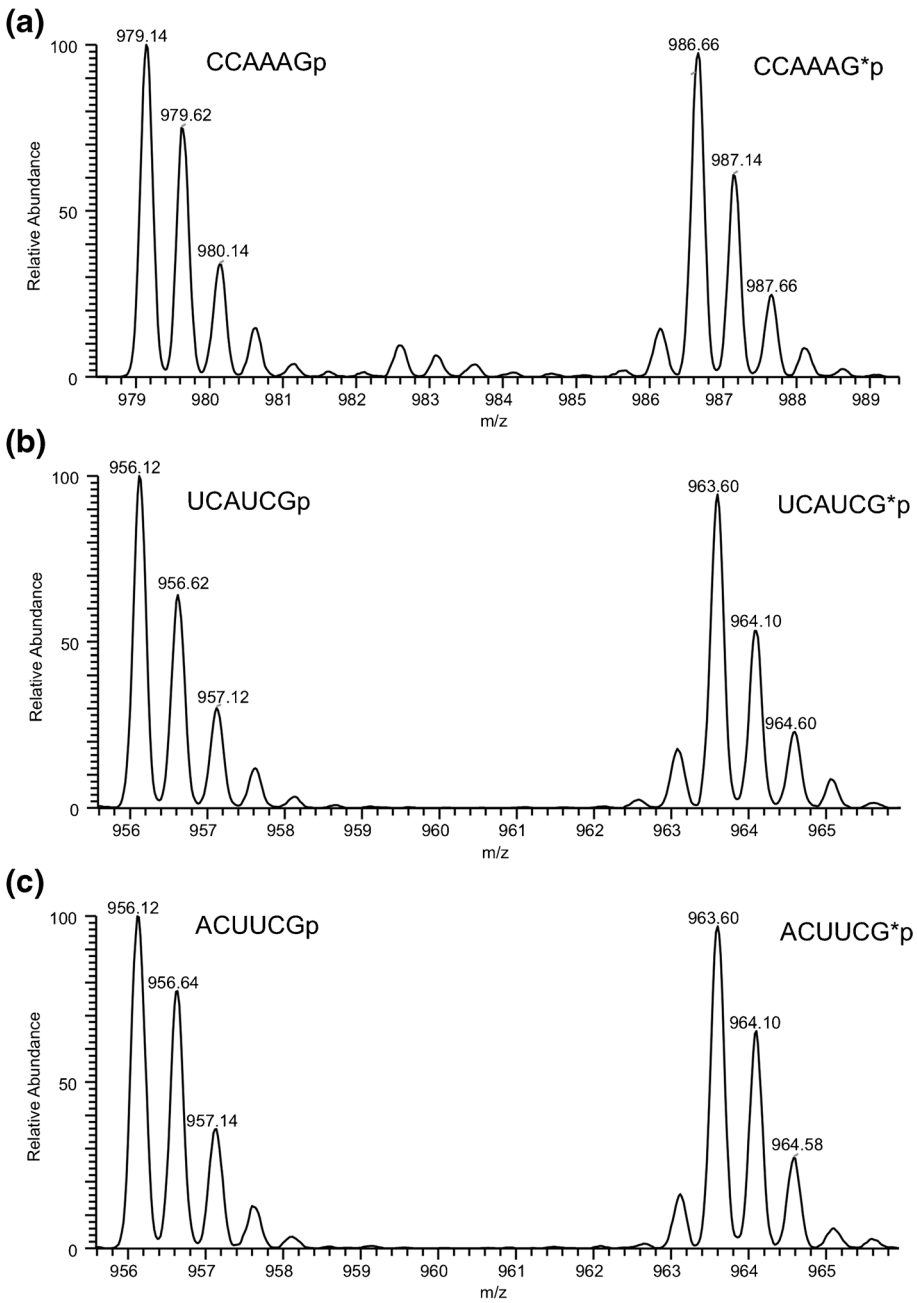


Figure 3. Mass spectral data for the *E. coli* tRNA Tyr II RNase T1 digestion products **(a)** CCAAAGp, **(b)** UCAUCGp, and **(c)** ACUUCGp. As the digestion products from reference and sample co-elute, the reference can be used for quantitative analysis of tRNA abundance in the mass spectrum

slight positive bias towards the isotopically labeled digestion products.

Similar curves were constructed for the eight other RNase T1 digestion products (data not shown) with all being linear with slopes around 1.0. Thus, a linear relationship of the monoisotopic ion abundance and concentration is established within one order of magnitude change in molar concentration, which is similar to the dynamic range obtained in $^{18}O/^{16}O$ labeling previously reported [34]. Given this linear response, it was informative to then review the earlier data obtained from the *E. coli* tRNA Tyr II standard and the isotopically labeled transcript. The measured ion abundances for the three doublets correspond to molar concentration ratios of the unmodified digestion products in the sample tested Thus, SIL-CARD is a straightforward and rapid means to quantify RNA digests. For accurate quantitative measurement, a calibration curve of the unknown sample with a known amount of internal standard is required, provided the response factor of the instrument does not deviate significantly from unity. Given the approach relies on comparing ratios of ion abundances, as long as the amount

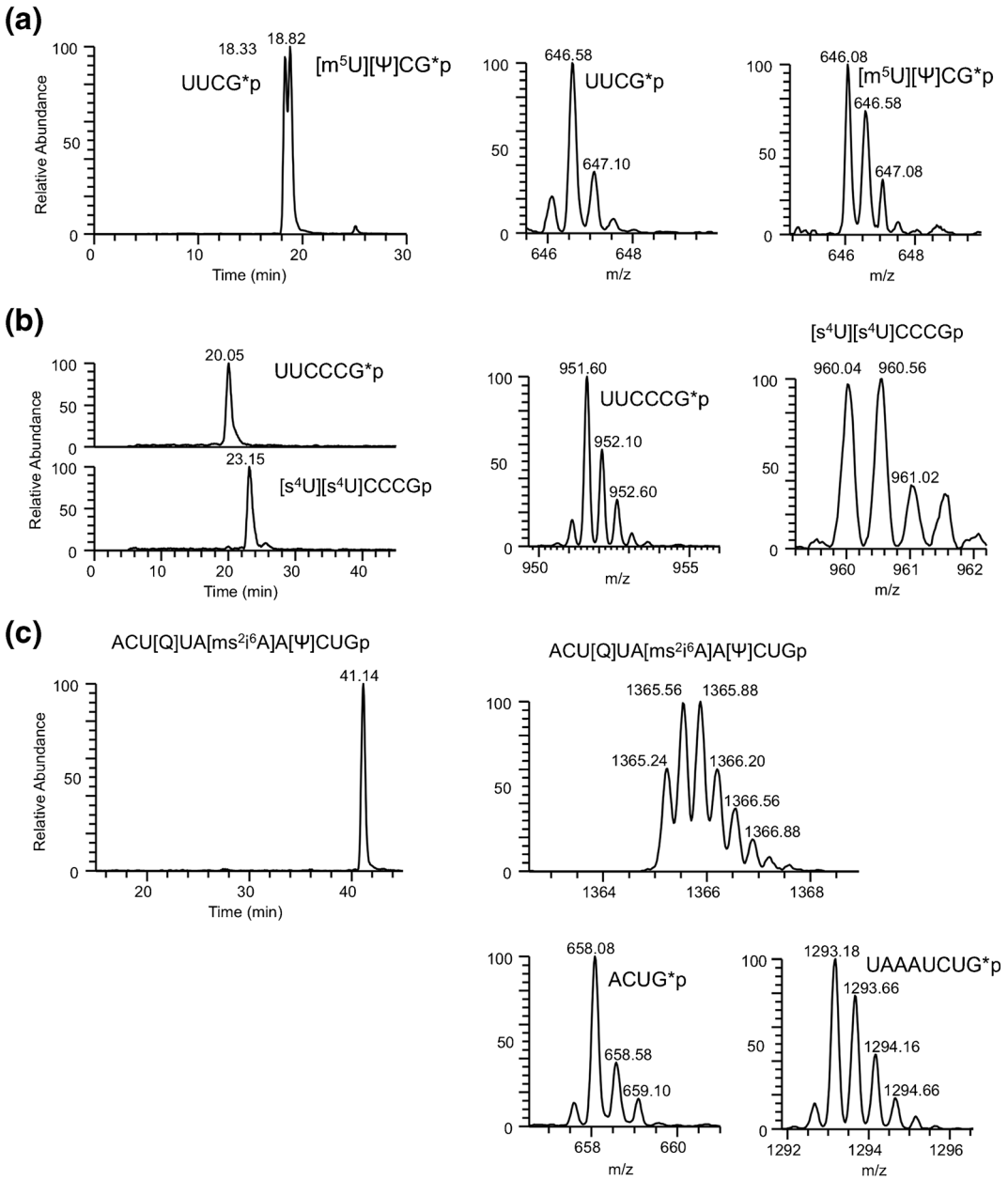


Figure 4. Extracted ion chromatograms (XICs) and mass spectra of the three expected singlets from SIL-CARD analysis of *E. coli* tRNA Tyr II, where the digestion products from the post-transcriptionally modified sample all elute after the tRNA transcript. **(a)** [m^5U]ΨCGp (*m/z* 646.08, –2) and UUCG*p (*m/z* 646.58, –2); **(b)** [s^4U][s^4U]CCCGp (*m/z* 960.04, –2) and UUCCCG*p (*m/z* 951.59, –2); **(c)** ACU[Q]UA[ms2i6A]A[Ψ]CUGp (*m/z* 1365.56, –4), ACUG*p (*m/z* 658.08), and UAAAUCUG*p (*m/z* 1293.18). The unmodified anticodon sequence (ACUGUAAAUCUGp) was not detected in the sample; hence no doublets for ACUGp and UAAAUCUGp are detected. Modified nucleosides: s^4U: 4-thiouridine; Q: Queuosine; ms^2i^6A: 2-methylthio-N^6-isopentenyladenosine; Ψ: pseudouridine; m^5U: 5-methyluridine

of internal standard is known with confidence, absolute quantification is possible. Because the detection and quantification is performed simultaneously, heavy transcripts could serve as an internal standard to follow changes in levels of particular RNase T1 digestion products.

Implicit in using ion abundances of RNase digestion products as a means of quantifying sample components is the expectation that the two oligonucleotides or RNAs will be digested equivalently. The most common ribonuclease used for RNA modification mapping is RNase T1. This enzyme is known to cleave at all unmodified guanosine residues with over-digestion leading to partial-digestion at adenosine residues [35]. The only modified guanosine that is recognized by RNase T1 is N^2-methylguanosine [36]. Thus, by using well-defined digestion conditions [35], equivalent digestion of two samples can be achieved. However, as other ribonucleases are

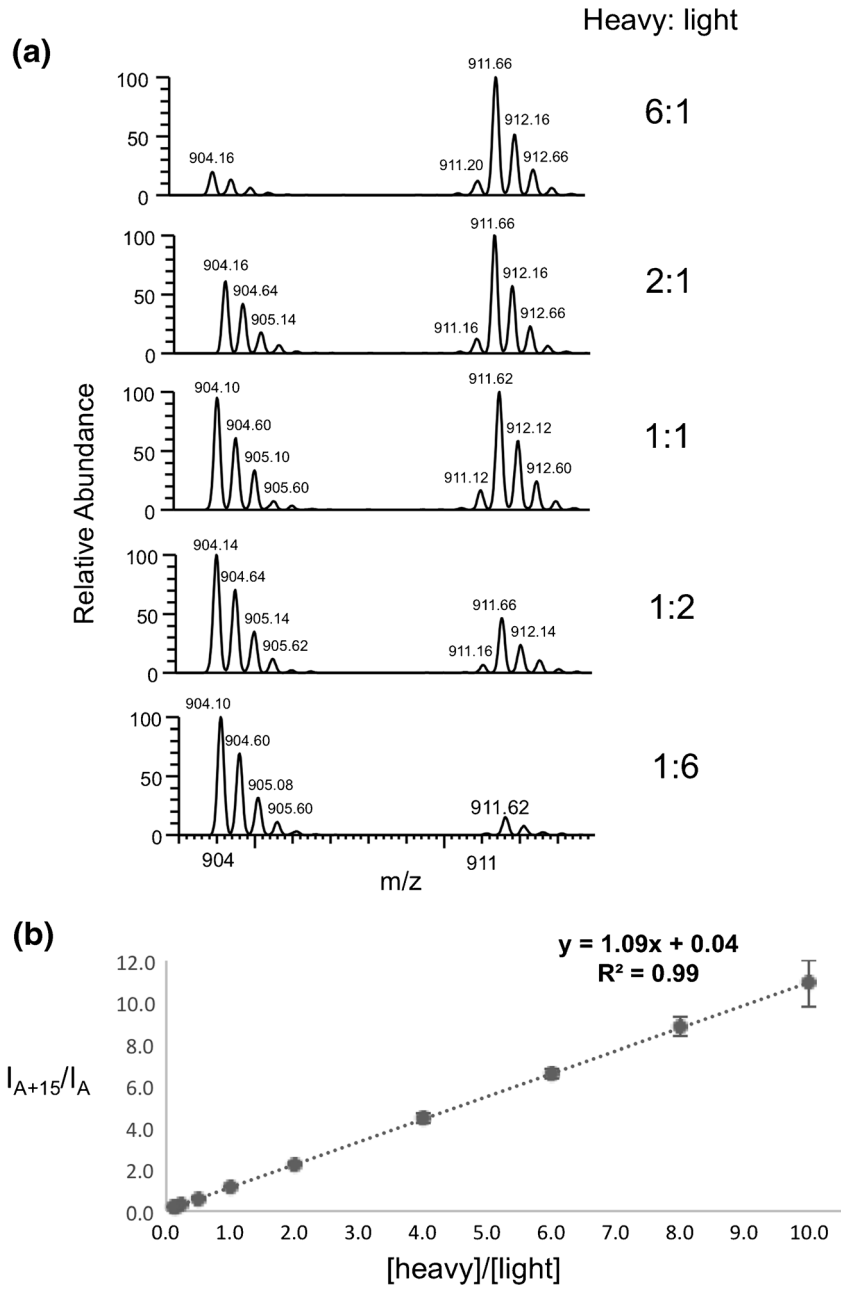


Figure 5. **(a)** Representative mass spectra of various heavy:light ratios for the RNase T1 digestion product UUCCCG. **(b)** Calibration curve generated for the ten different heavy:light ratios of UUCCCG listed in Table 2

now being used for RNA modification mapping [14, 37], it will be important to verify no differential digestion behavior before pursuing quantitative applications.

Benefits of SIL-CARD for tRNA Characterization

An overall evaluation of the data obtained using isotopically labeled in vitro tRNA transcripts as the reference for CARD finds several important advantages compared with the prior approach based on the enzymatic incorporation of $^{18}O/^{16}O$ during RNase digestion. *Spectral congestion is reduced.* Overlapping isotope peaks, due to the natural ^{13}C and ^{15}N present in the RNAs, makes data analysis quite challenging when searching singlets and doublets. SIL-CARD reduces spectral congestion by increasing the mass shift from +2 Da to +15 Da (for guanosine). Moreover, even long RNase digestion products, which may be detected at relatively high charge states, can still be differentiated by SIL-CARD (Figure 6). *The labeling step is separated from enzymatic treatment.* The prior iteration required isotopic labeling by the complete hydrolysis of RNA in heavy water. However, not all RNases uniformly generate 3′-phosphates as some RNases (i.e., U2, A, cusativin), halt at cyclic phosphate intermediates. As these intermediates cannot incorporate the $^{18}O/^{16}O$ label, a second round of acid or

Table 2. Data Obtained and Used to Generate the Calibration Curve Shown in Figure 5b. The Experimental Responses were Obtained from the Monoisotopic Ion Abundances (in arbitrary units) from the Oligonucleotide UUCCCG in Both Labeled and Unlabeled Form

[heavy]/[light]	Calculated average monoisotopic ion abundance ratio[a]	% CV
10.0	10.9 ± 1.1	8.30
8.00	8.83 ± 0.45	9.43
6.00	6.56 ± 0.21	8.60
4.00	4.44 ± 0.23	9.82
2.00	2.25 ± 0.062	10.9
1.00	1.18 ± 0.019	15.1
0.500	0.580 ± 0.039	13.6
0.250	0.270 ± 0.007	11.9
0.167	0.200 ± 0.011	16.1
0.125	0.140 ± 0.002	13.1

[a]n = 3, ±SD

enzymatic treatment using lambda phosphatase (LPP) to transform cyclic phosphates to linear products is required for labeling [38]. By separating the labeling step from any enzymatic digestion steps, the label will always be present in SIL-CARD (Figure 2). *Labeled transcripts enable broader applicability of the method.* Unlike earlier versions of CARD, which are limited to using cellular RNAs, the modification status of which is fully characterized as references, the use of in vitro transcripts as references means that this method can be applied to any tRNA or tRNA pool so long as the tDNA sequences are known. The much greater availability of genomic sequence information over RNA sequence information with all modifications annotated should enable this approach to be used in a wide variety of assays, including those that would monitor tRNA modification profiles in response to different external stimuli [8, 10].

Conclusions

The use of isotopically enriched RNA transcripts, previously applied to the analysis of modified rRNA, is adapted here for the comparative analysis of tRNA digests. Unlike prior iterations of CARD, SIL-CARD simplifies the identification of doublets and inherently incorporates an isotopically labeled standard suitable for quantitative analysis of tRNA abundance.

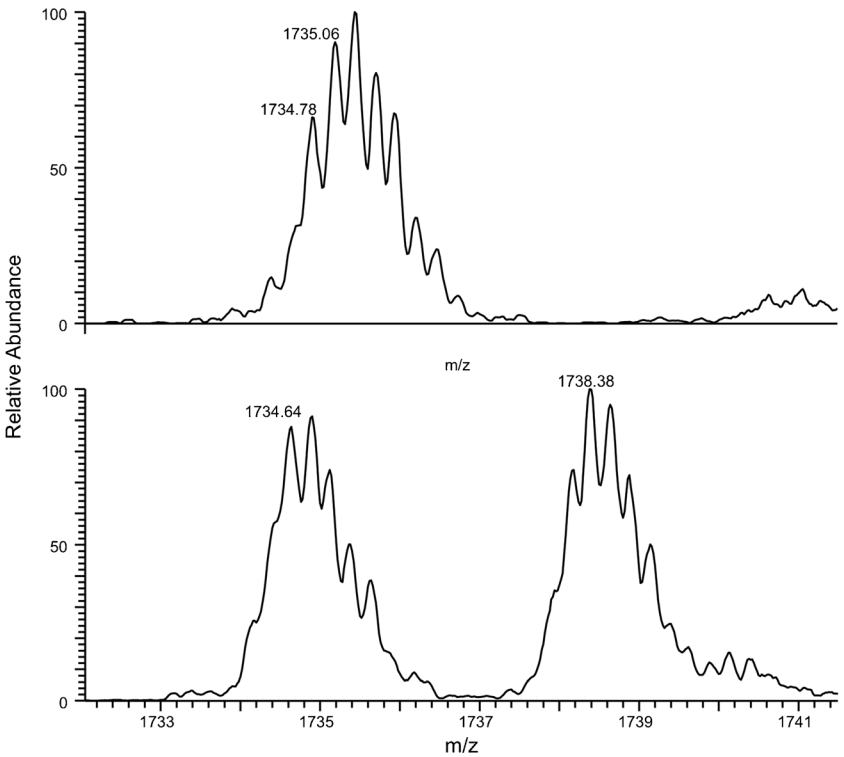


Figure 6. Mass spectra of the unmodified oligonucleotide AAUCCUUCCCCCACCACCAAAGp (*m/z* 1734.78, –4 charge state). **(a)** When this oligonucleotide is labeled by the ^{18}O approach, the predicted doublet (*m/z* 1734.78 and 1735.06) is not readily identified at this high charge state. **(b)** When the same oligonucleotide is labeled by using enriched GTP, the expected doublet can easily be identified with a low resolution mass analyzer

Although the proof-of-concept work presented here is limited to a single tRNA, the method is scalable to tRNA mixtures in the same manner as traditional CARD. This improved method should prove particularly useful for studies focused on dynamic changes in tRNA modification levels in addition to more conventional characterization of tRNA modification profiles.

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