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Characterization of Post-Transcriptional RNA Modifications by Sheathless Capillary Electrophoresis−High Resolution Mass Spectrometry

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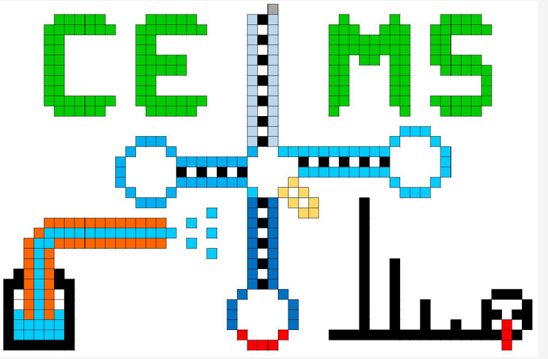
ABSTRACT: Over the past decade there has been a growing interest in RNA modification analysis. High performance liquid chromatography−tandem mass spectrometry coupling (HPLC−MS/MS) is classically used to characterize post-transcriptional modifications of ribonucleic acids (RNAs). Here we propose a novel and simple workflow based on capillary zone electrophoresis−tandem mass spectrometry (CE−MS/MS), in positive mode, to characterize RNA modifications at nucleoside and oligonucleotide levels. By first totally digesting the purified RNA, prior to CE−MS/MS analysis, we were able to identify the nucleoside modifications. Then, using a bottom-up approach, sequencing of the RNAs and mapping of the modifications were performed. Sequence coverages from 68% to 97% were obtained for four tRNAs. Furthermore, unambiguous identification and mapping of several modifications were achieved.



All the classes of RNAs are post-transcriptionally modified. RNA modifications increase the structural and functional diversity of RNA and as a consequence influence RNA structural stability, RNA base pairing, and protein recognition. There is little information regarding RNA modifications. To increase the knowledge of the community, it is important to use sensitive approaches. Several analytical methods have been developed to study RNA modifications. Among them, RNA-seq techniques are the most common approach to locate RNA modifications. However, this method can be limited for the characterization of certain modifications due to the blockage mechanism.[1,2] In parallel, mass spectrometry (MS) has increasingly become a valuable technique to investigate modifications because it provides specificity and sensitivity and may also perform structural elucidations.[3] In recent decades, strategies using enzymatic digestions, prior MS detection, were established to study RNA.[4] However, despite the latest impressive improvements of MS in terms of resolution and sensitivity, MS often needs to be hyphenated to separation methods to perform deeper characterization of biomolecules. Since the report of electrospray ionization (ESI), HPLC−ESI−MS/MS has rapidly become an important and popular tool for the investigation of nucleic acid modifications. Indeed, this tool can be used not only to characterize modifications but also to quantify them.[1,5−8] It permitted the discovery of many unknown RNA modifications which, to date, represent more than 160 natural chemical modifications reported in the literature.[9] Capillary zone electrophoresis (CZE) is a complementary method to liquid chromatography. In CZE, analytes are separated under an electrical field which offers different selectivity. In addition, CZE has the major advantages of low sample consumption and fast and effective separations. CE equipped with optical detection were abundantly reported to study RNA at different levels.[10−13] However, no structural information can be extracted from this type of detector and/or standards should be used to identify the modifications.[14−16] Hyphenation of CE−MS was a long time challenge due to the necessity to maintain two independent voltages (separation voltage and electrospray voltage). Since its first report in the late 80s by Olivares et al.,[17] CE coupled with MS became highly interesting for the analysis of biologics.[18] With regard to RNA analysis, Feng et al. developed a pressure assisted electrokinetic injection technique to measure oligonucleotides and their adducts in 2007.[19] In 2016, Khan et al. reported the use of CE−MS to directly characterize modifications of microRNAs in cancer serum.[20] CZE coupled to MS was also used to estimate the rate and equilibrium constant of the conformational dynamics of DNA G-Quadruplex.[21] More recently, Demelenne et al. performed CZE−MS analysis to characterize therapeutic oligonucleotides.[22] Different geometries of the interface were developed

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since the first CE−MS hyphenation.[23] In 1997, Moini et al. developed a sheathless interface.[24] The design of this interface has the ability to generate a nanoESI and provides excellent sensitivity.[25] Indeed, several articles reported the use of this sheathless interface in many fields of application, such as metabolomic,[26] proteomic,[27] or glycomic.[28,29] RNA modifications are chemical changes that generally lead to a mass shift and/or charge shift. Recently, Yu et al. reported the first study of the quantification of eight RNA modified nucleotides by multiple reaction monitoring (MRM) using CZE−ESI−MS/MS.[30] In this work, a simple workflow based on CZE−ESI−MS/MS was developed to characterize RNA post-transcriptional modifications at two different levels using the same CZE experimental conditions. Although RNA are classically detected in the negative ion mode, in the present study, detection of analytes was achieved in the positive ion mode. In this approach, we performed a total hydrolysis of RNA to screen modifications at the nucleoside level, and then, a bottom-up approach was developed with the aim of sequencing RNA and mapping the modifications at the oligonucleotide level. The implemented strategy allowed us to highlight the modifications present on the RNAs but also to locate them on the primary structure. For this study, four purified and desalted tRNAs from *S. cerevisiae* ($tRNA^{Asp}_{GUC}$; $tRNA^{Ala}_{AGC}$; $tRNA^{Trp}_{CCA}$; and $tRNA^{Phe}_{GAA}$) were selected as model molecules. These tRNAs contain about 75 nucleic acids, and their sequences and modifications (type and location) are well-known and have already been described in the literature.[9,31]

MATERIALS AND METHODS

Chemicals and Reagents. The chemicals used were of analytical grade or high purity. The water used to prepare the samples solutions or buffers was obtained using a Direct-Q3 UV-R water purification system (Merck Millipore, Amsterdam, The Netherlands). Bacterial Alkaline Phosphatase (BAP) (150 U/μL), RNase T1, and RNase A were purchased from ThermoFisher Scientific (Marietta, OH, U.S.A.). RNase P1 from Penicillum citrinum was purchased from Sigma−Aldrich (Saint Louis, MO, U.S.A.)

Sample Preparation. Purified tRNAs from *S. cerevisiae* ($tRNA^{Asp}_{GUC}$; $tRNA^{Ala}_{AGC}$, $tRNA^{Trp}_{CCA}$; and $tRNA^{Phe}_{GAA}$) were prepared and desalted by three successive precipitations with ammonium acetate. For nucleoside analysis, tRNAs were diluted to a concentration of 2 μg/μL in H_2O. Digestion was carried out in this order: 7 μL H_2O; 1 μL buffer P1 10× (2 mM $ZnCl_2$, 250 mM NH_4OAc, pH5.0); 1 μL of tRNA; and 1 μL of P1 (0.5 U/μL). The mixture was incubated at 37 °C for 2 h. After P1 digestion, 10 μL of BAP (1.5 U/μL in 100 mM NH_4OAc) was added to the mixture and incubated at 37 °C for 4 h. The mixture was diluted twice in NH_4OAc 200 mM pH 4 and analyzed by CE−MS/MS. For the oligonucleotide analysis, tRNAs were diluted to a concentration of 0.75 μg/μL. For RNase T1 digestion, a solution of 100 μL of RNase T1 (1/1000 in NH_4OAc100 mM) + 0.5 μL of BAP (150 U/μL) was made. Ten μL of this solution +1 μL of tRNA were mixed, and the samples were incubated overnight at 37 °C. Then the mixture was directly analyzed by CE−MS/MS. For RNase A digestion, a solution of 1000 μL of RNase A (1/1000 in NH_4OAc 100 mM) + 0.5 μL of BAP (150 U/μL) was made. Ten μL of this solution +1 μL of tRNA were mixed and samples were incubated overnight at 37 °C. Then, the mixture was directly analyzed by CE−MS/MS.

Capillary Electrophoresis. All of the CE experiments have been done on a CESI8000 capillary electrophoresis system from Sciex Separation (Brea, CA, U.S.A.). The 32 Karat (Sciex separation) software was used for instrument control and data acquisition. Samples were stored at 7 °C in a sample tray before injection. Bare fused-silica capillary (total length 100 cm; 30 μm i.d.) with porous tip on its final end, a second capillary filled during experiments with BGE (10% acetic acid) allows electrical contact. Conditioning of new capillaries was performed by applying 10 min 50 psi of NaOH 1 M, NaOH 0.1 M, and H_2O. For CE experiments, hydrodynamic injection (4 psi for 9.1 s) corresponding to a total volume of 5 nL of sample injected was used (for total and partial digestions). Separations were achieved applying +30 kV. After each run, the capillary was rinsed by applying 1 min 100 psi of MeOH, H_2O, NaOH 0.1 M, H_2O, HCl 0.1 M, H_2O, and 2 min 100 psi of BGE.

Mass Spectrometry. For nucleoside analysis, the CE system was coupled to a maXis 4G mass spectrometer (Bruker, Bremen, Germany) by the sheathless interface. The maXis MS is equipped with a hybrid analyzer composed of quadrupoles followed by time-of-flight (TOF). Otof control 3.4 software allowed to pilot the nanoESI source. The source settings were as follows: Ion polarity, positive; nanoESI voltage, + 1500 V; source heating temperature, 130 °C; dry gas, 1.2 L/min; and mass/charge (m/z) range, 80−800 for MS and MS/MS detection. Gas-phase fragmentations were performed using N_2. Collision energies were set at 20 eV for CID experiments. For oligonucleotide analysis, the same source parameters were used except for the m/z range which was 230−2300 for MS and MS/MS detection. Gas-phase fragmentations were performed using N_2. Collision energies for CID experiments ranged from 6 to 48 eV according to the m/z ratio of the precursors.

Data Analysis. Data obtained from CE−MS/MS experiments were analyzed using DataAnalysis software (Bruker, Bremen, Germany). All data were manually interpreted. Sequences of tRNA as well as locations of the structures of the modified nucleotides were found in the Modomics database (https://iimcb.genesilico.pl/modomics/). This database is constantly verified and regularly updated. m/z ratios of theoretical digest products were simulated using MongoOligo mass calculator v2.08 (https://mods.rna.albany.edu/masspec/Mongo-Oligo). The m/z ratios of the fragment ions generated by CID experiments were also calculated by MongoOligo. It is important to note that RNase T1 and RNase A cleave RNA after specific nucleotides, but could also cleave after certain modified nucleotides. For example, RNase T1 cleaves after m2G and RNase A cleave after D and m5U. MongoOligo simulator (v2.08) does not cleave modifications and the simulated fragments from this calculator should be checked.

For the analysis of total hydrolysis, the theoretical m/z of the unmodified and modified compounds were extracted with a tolerance of ±0.02 m/z. Identification of compounds was performed using the migration times, precursor ions, and product ions.

For the analysis of oligonucleotides, the theoretical m/z of compounds were extracted with a tolerance of ±0.02 m/z. Identifications of digested RNA fragments were done from CID spectra by reconstructing the sequence following y and/or c series. If this is not possible, then other series can be used. Modifications are localized with the same procedure. Then the experimental results were confronted with the theoretical ones.

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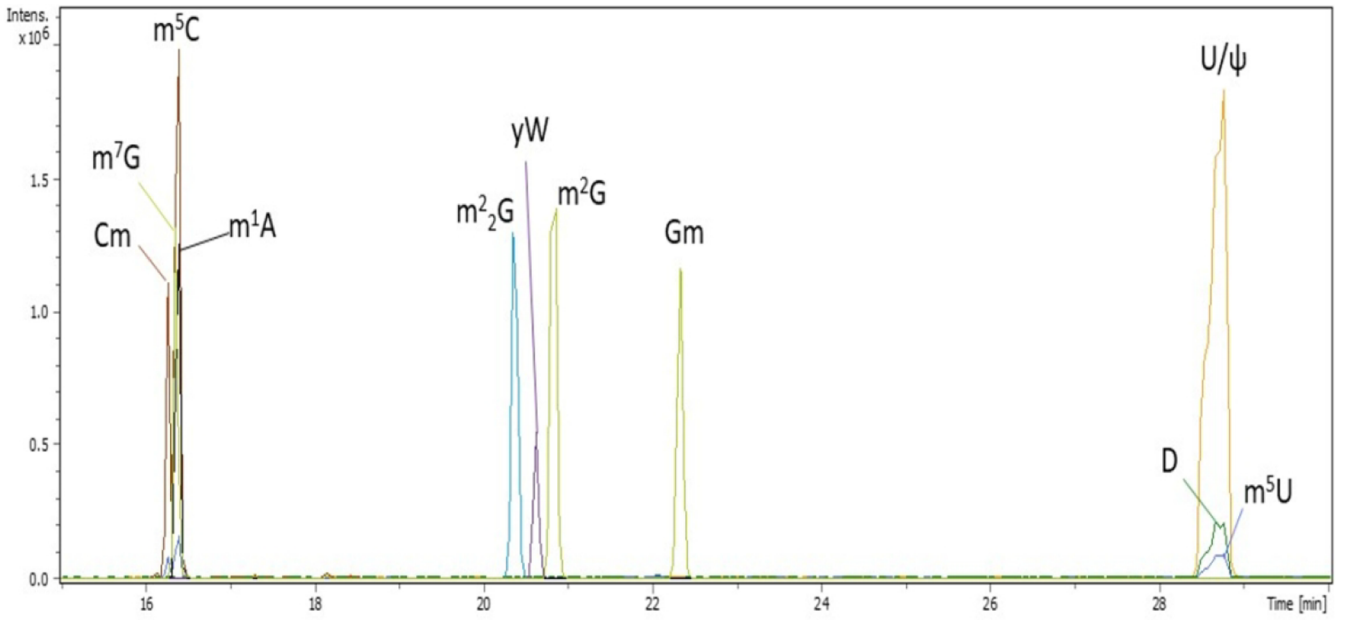

Figure 1. Multiple extracted ion electropherograms for the analysis of RNase P1 BAP digested $tRNA^{Phe_{GAA}}$ from *S. cerevisiae* by CE−MS in positive ion mode, in which only modified bases are extracted. Injected volume 5 nL (10 fmol).

Table 1. List of All Digested Products Experimentally Observed for $tRNA^{Phe_{GAA}}$, after Digestion with RNase P1 BAP and CE−MS/MS Analysis

precursor ion (m/z)	product ion (m/z)	molecular mass (Da)	migration time (min)	assigned nucleoside
244.09	112.05	243.09	16.0	C
245.08	113.04	244.07	28.7	U
268.10	136.06	267.10	16.5	A
284.10	152.06	283.09	21.9	G
298.12	166.07	297.11	20.9	m^2G
247.09	115.05	246.08	28.7	D
312.13	180.09	311.12	20.4	$m^2{}_2G$
258.11	112.05	257.10	16.3	Cm
298.12	152.06	297.11	22.3	Gm
509.19	377.16	508.19	20.6	yW
245.08	125.04	244.07	28.7	Ψ
258.11	126.07	257.10	16.4	m^5C
298.12	166.07	297.11	16.4	m^7G
259.09	127.05	258.08	28.7	m^5U
282.12	150.08	281.11	16.4	m^1A

RESULTS AND DISCUSSION

Characterization at the Nucleoside Level. To study RNA modifications, classical strategies based on different ribonucleases (RNases) were assessed.[5,7] The first level of characterization consisted to perform the total hydrolyses of the sample with the aim of identifying modified nucleotides present in the RNAs. This step was performed using ribonuclease P1 (RNase P1) which cleaves 3′ phosphate of any nucleotide. The remaining 5′ phosphates were removed using bacterial alkaline phosphatase (BAP) to obtain a mixture of nucleosides. A 30 min separation of ribonucleosides was achieved by applying +30 kV with a background electrolyte constituted of 10% acetic acid (pH 2.2) and a sample volume of 5 nL (corresponding to 10 fmol injected).[30] While RNA are classically detected in negative ion mode due to their acidic property, exploring the positive mode can provide another facet of RNA analysis and thus could show complementarity. To screen modified nucleosides, the analytes were then online detected by CZE−ESI−MS/MS in positive ion mode. Extracted Ion Electropherograms of nucleosides from $tRNA^{Phe_{GAA}}$ are shown in Figure 1 (for other tRNA, see Supporting Information, SI, Figures S1−S3). As expected, the four canonical nucleosides and modified nucleosides were separated and characterized with ±0.02 m/z. To increase identification confidence, MS/MS of nucleosides was performed. For peaks assignments, identifications were confirmed when specific transitions were detected.[32] For example, in the case of uridine and pseudouridine, these species were identified using MS/MS spectra because these isomers have different fragmentation patterns. However, these methods cannot distinguish nucleosides having the same fragmentation pattern. In that case, standards need to be used to characterize modification based on their migration times. For $tRNA^{Phe_{GAA}}$, all the known modifications described in the Modomics database were detected and compiled in Table 1 (for other tRNA, see SI Tables S1−S3). The most important and

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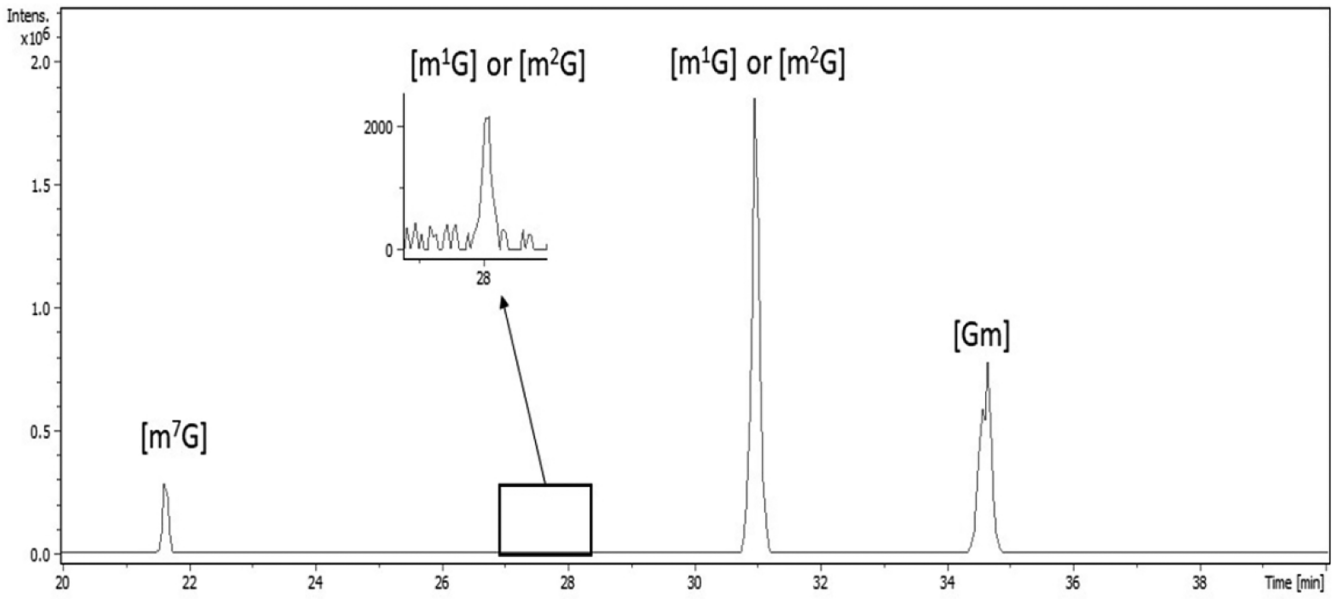

Figure 2. Extracted ion electropherograms of 298.12 m/z for the analysis of RNase P1 BAP digested $tRNA^{Trp_{CCA}}$ by CE−MS/MS in positive ion mode. Injected volume 5 nL (10 fmol).

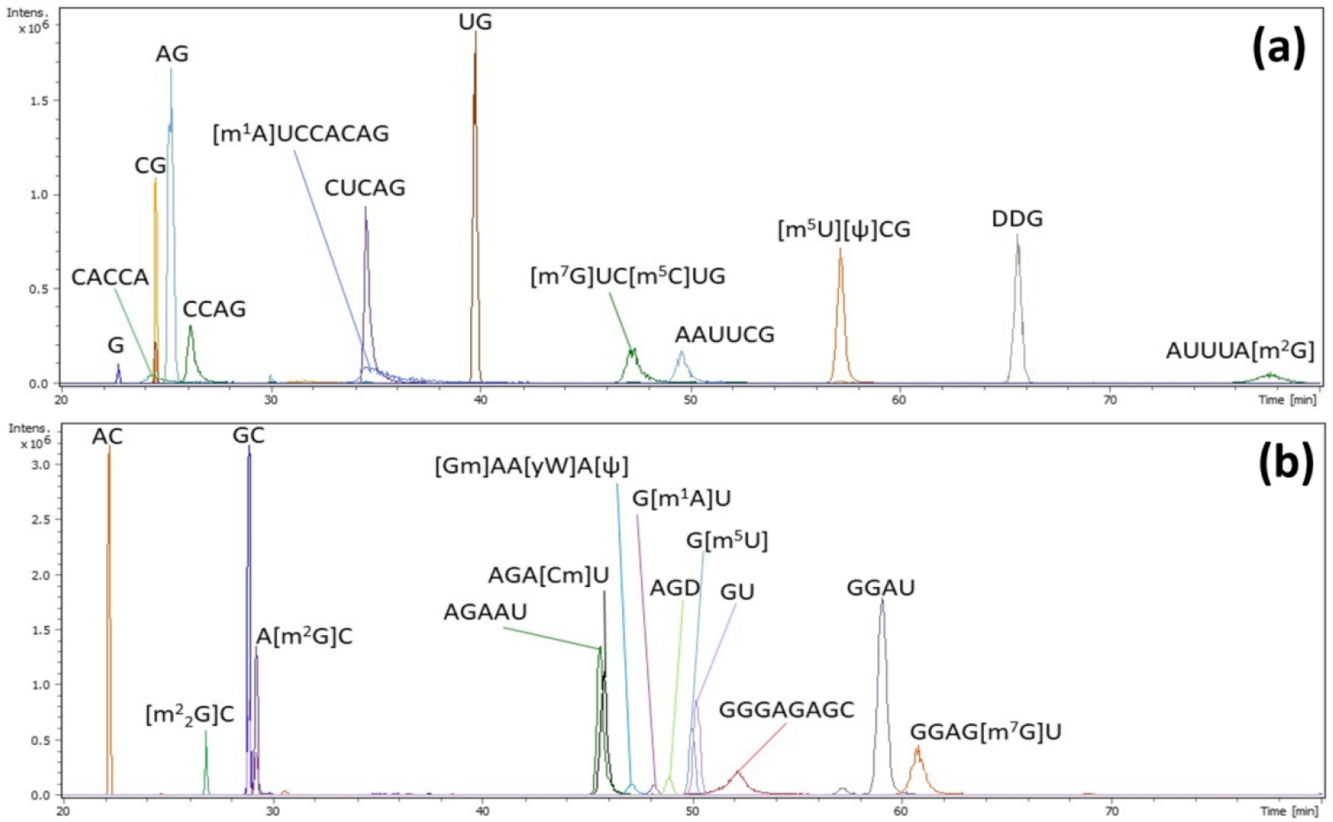

Figure 3. Multiple extracted ion electropherograms for the analysis of (a) RNase T1 BAP and (b) RNase A BAP digested $tRNA^{Phe_{GAA}}$ by CE−MS in positive ion mode. Injected volume 5 nL (10 fmol).

common modified nucleosides were identified as dihydrouridine (D) and methylated nucleosides; however, in the same set of data, other more complex modifications such as wybutosine (yW) were also characterized. Interestingly, in the case of $tRNA^{Trp_{CCA}}$ which contains four methylated guanosines (1-methylguanosine m^1G; N2-methylguanosine m^2G; 2′-*O*-methylguanosine Gm and 7-methylguanosine m^7G), separation of these isomers were achieved using CZE−ESI−MS/MS as shown in Figure 2. The excellent separation provided by CZE enables the best selectivity for these isomers. While Gm was characterized with its different fragmentation pattern and m^7G with the analysis of P1

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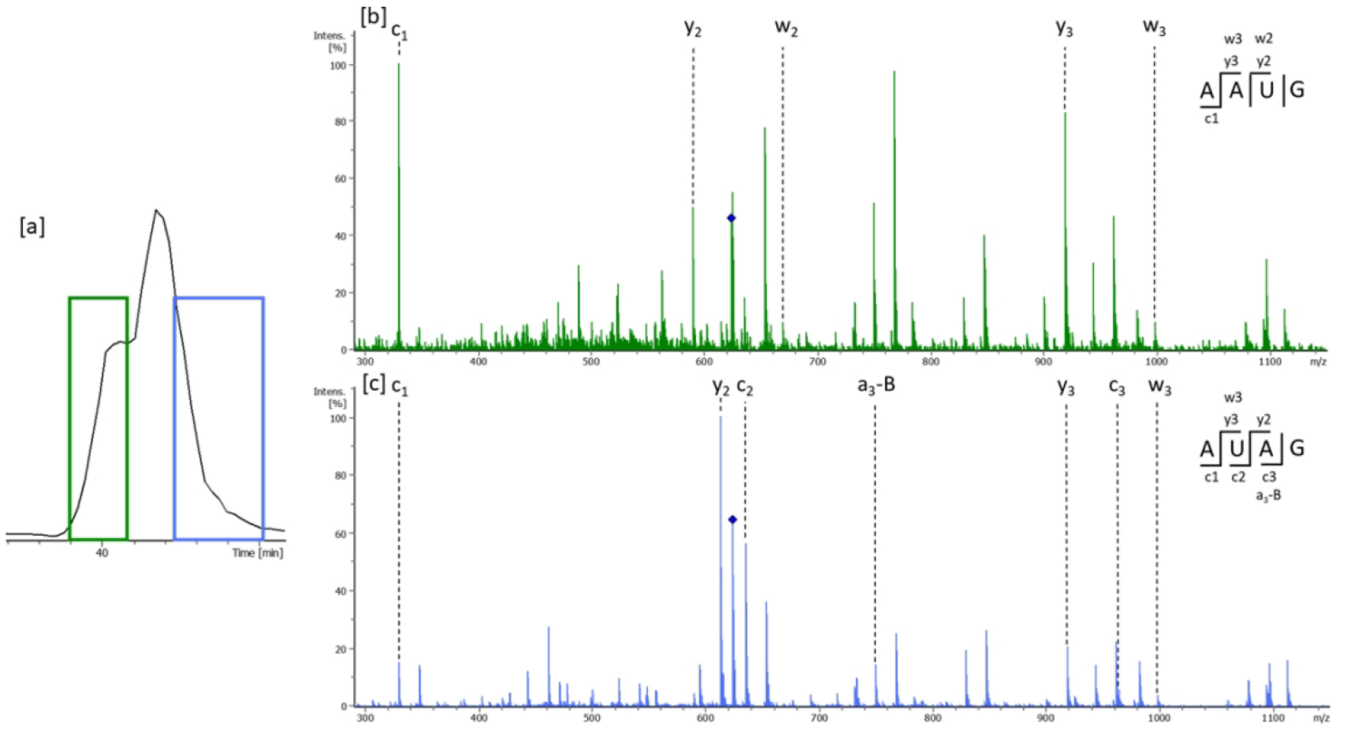

Figure 4. [a] Extracted ion electropherograms of m/z 624.2 corresponding to the $tRNA^{Asp}_{GUC}$ RNase T1 digestion products AAUG and AUAG. [b] CID mass spectrum of m/z 624.21 @ 39.9 min. [c] CID mass spectrum of m/z 624.21 @ 40.4 min.

digested tRNA Val from the *S. cerevisiae* standard (data not shown), m^1G and m^2G were not assigned because the standards were not available. Thus, we demonstrated the ability of CZE−ESI−MS/MS to easily screen and identify all modifications contained in the four tRNA. However, this level of characterization does not allow one to locate modifications on RNA sequences.

Characterization at the Oligonucleotide Level. Hence, a strategy was implemented to map modified nucleotides on the RNA sequence. In addition to the location of previously identified modifications, primary structural characterization was simultaneously assessed using the same set of data. For this purpose, a bottom-up approach was developed using exactly the same CZE−ESI−MS/MS conditions as those used for ribonucleoside characterization. On the basis of a proteomic-like strategy, RNase digestions have been performed to obtain a pool of short oligonucleotides. To increase the sequence coverages, a workflow based on two specific digestions was realized. Prior CZE−ESI−MS/MS analysis, samples were individually digested by RNase T1 and RNase A.[33,34] RNase T1 cleaves specifically at the 3′ end of guanosine while RNase A cleaves specifically at the 3′ end of pyrimidines. The major advantages of these RNases are their strong specificity and their excellent reactivity involving the no production of miscleavages. In addition, dephosphorylation of terminal phosphates using BAP was also performed simultaneously to each digestion to improve separation (data not shown) and facilitate MS/MS spectral interpretation.[35] Following the digestion step, oligonucleotides were analyzed in 60 to 80 min by CZE−ESI−MS/MS in positive polarity using the same experimental conditions described for the ribonucleosides study. The similarity of the experimental conditions is a very important advantage in terms of analysis time because it allows one to perform the two levels of characterization without any instrumental and experimental modification. In comparison with LC−MS workflows, analysis of nucleosides and oligonucleotides have different experimental conditions (column, nature of mobile phase, etc.), which considerably complicates the implementation of RNA characterization at different levels and consequently increases the analysis time. Following the CZE−ESI−MS detection, fragmentation of oligonucleotides by MS/MS experiments were achieved using collision-induced dissociation (CID). Presently, software for data treatment of MS and MS/MS spectra of RNA were developed (RNAModMapper,[36] RoboOligo,[37] or Ariadne[38]), however, they are not yet available in positive mode. Then, we carried out the manual interpretation of MS/MS raw data to avoid errors for unambiguous mapping of modifications. Excellent separations of RNase T1 and RNase A oligonucleotides were achieved by CZE−ESI−MS/MS for each tRNA, facilitating significant MS spectral interpretation. As an example, the extracted ion electropherogram of the oligonucleotides is shown in Figure 3 for $tRNA^{Phe}_{GAA}$. (For other tRNA, see SI Figures S4−S9). MS/MS spectra were also manually interpreted. The primary structures were reconstructed following the y and/or c series.[39] a-B and w ions can also be useful if the sequencing is difficult or to confirm the sequence. To validate the oligonucleotides, we have considered only the those detected in MS and fully sequenced by MS/MS. For the larger ones, we often get MS spectra but sometimes they were not abundant enough to be fragmented. Therefore, it drops the sequence coverage. It is also important to note that specific RNases produce mononucleotides or dinucleotides that are generally not sequence specific, hence diminishing the sequence recoveries. In our study, only unique mono- and dinucleotides were considered for sequence recoveries. Isobaric species also represent a common issue for RNA sequencing. For example, in the case of RNase T1 hydrolysis of

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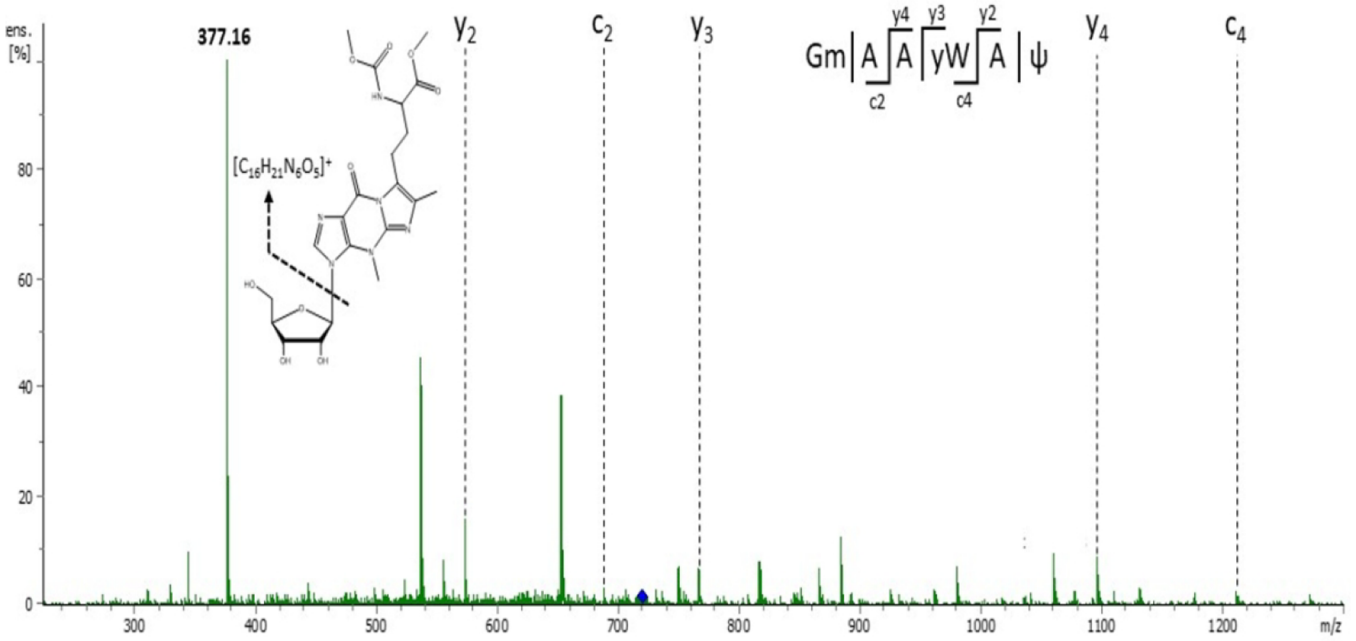

Figure 5. CID mass spectrum of RNase A product [Gm]AA[yW]A[ψ] (m/z 721.15) from *S. cerevisiae* $tRNA^{Phe}_{GAA}$ with fragmentation assignments. Peak of m/z 377.16 correspond to the loss of the modified base yW.

Table 2. List of All Digested Products Experimentally Observed for $tRNA^{Phe}_{GAA}$, after Individual Digestion with RNase T1 and RNase A[a]

theoretical (m/z)	experimental (m/z)	z	molecular mass (Da)	migration time (min)	oligonucleotide
RNase T1					
589.14	589.14	+1	588.14	24.6	CG
937.65	937.64	+2	1873.29	77.6	AUUUA[m^2G]
765.13	765.13	+2	1528.25	34.6	CUCAG
900.18	900.17	+1	899.18	65.6	DDG
612.12	612.12	+2	1222.23	26.3	CCAG
940.16	940.15	+2	1878.30	47.3	[m^7G]UC[m^5C]UG
590.12	590.12	+1	589.12	39.8	UG
608.11	608.10	+2	1214.20	57.2	[m^5U][ψ]CG
836.15	836.14	+3	2505.40	35.3	[m^1A]UCCACAG
930.15	930.14	+2	1858.29	49.6	AAUUCG
756.64	756.64	+2	1511.28	24.3	CACCA
RNase A					
632.62	632.61	+2	1263.22	59.1	GGAU
932.21	932.20	+1	931.21	29.3	A[m^2G]C
461.10	461.10	+2	920.19	49.0	AGD
876.48	876.48	+3	2626.43	52.2	GGGAGAGC
617.17	617.17	+1	616.17	23.6	[$m^2{}_2$G]C
784.15	784.14	+2	1566.28	45.9	AGA[Cm]U
721.15	721.15	+3	2160.44	47.1	[Gm]AA[yW]A[ψ][1]
984.67	984.67	+2	1967.33	60.9	GGAG[m7G]U
590.12	590.12	+1	589.12	50.3	GU
604.14	604.14	+1	603.13	50.0	G[m^5U]
933.19	933.19	+1	932.19	48.2	G[m^1A]U
789.14	789.14	+2	1576.27	45.7	AGAAU
268.10	268.11	+1	267.10	18.5	A
total sequence					
GCGGAUUUA[m^2G]CUCAGDDGGGAGAGC[$m^2{}_2$G]CCAGA[Cm]U[Gm]AA[yW]A[ψ][m^5C]UGGAG[m^7G]UC[m^5C]UGUG[m^5U][ψ]CG[m^1A]UCCACAGAAUUCGCACCA					

[a]Identified sequences are in bold. [1]MS/MS identification was done considering loss of m/z 376 corresponding to loss of modified base yW.

$tRNA^{Asp}_{GUC}$, fragments AUAG and AAUG were the products. The performances of the CE separation combined with the MS/MS spectral quality allow us to characterize both isobaric species without any ambiguity (Figure 4). Finally, compiling

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the two digestions, a sufficient number of sequence specific oligonucleotides were detected by MS and MS/MS to obtain the sequence recoveries from 68% to 97% for the studied tRNAs. Concerning RNA modifications, the same manual data treatment has been realized to locate modifications on the RNA sequence. The calculated m/z ratio corresponding to modified oligonucleotides have been extracted using MS spectra and sequenced using MS/MS spectra. Figure 5 shows an example of the MS/MS sequencing for the [Gm]AA[yW]-A[ψ] oligonucleotide. Here, the fragment of 377.15 m/z (z = 1) corresponded to the fragmentation of this modified nucleoside [yW]. Thus, loss of the modified base must be considered to sequence this oligonucleotide. Deduction of 376.15 Da was done for the fragment ions that contain the modified base and permitted the successful sequencing of this modified oligonucleotide. The MS/MS spectral quality allowed precise location of the modified nucleotides on the sequences. However, if CZE−ESI−MS/MS analysis of RNA digests enables us to determine the precise position on the RNA sequence, then in the case of methylation it does not allow us to locate the modification on the ribose and/or base. Furthermore, the list of the identified fragments by the CZE−ESI−MS/MS analysis is summarized in Table 2 for $tRNA^{Phe}_{GAA}$ (for other tRNA, see SI Tables S4−S6). We were able to locate a large number of modifications previously characterized at the nucleoside level, including the wybutosine modification on $tRNA^{Phe}_{GAA}$.

CONCLUSIONS

In summary, we developed a simple CZE−ESI−MS/MS workflow for the successful characterization of post-transcriptional RNA modifications. On the basis of strategies using three different ribonucleases, RNA sequencing and modification characterization have been performed at the nucleoside and oligonucleotide levels. Using the same platform with the same experimental conditions, we managed to obtain excellent sequence recoveries and were able to identify and map modifications onto primary sequences of tRNAs. CE−MS/MS could become a powerful tool for unambiguous chemical characterization and sequence placement of modifications onto tRNAs sequences (including mitochondrial tRNAs) with software support. Moreover, CE−MS/MS could be applied to other modified noncoding RNA, especially rRNAs.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.0c01345.

Figures S1−S9, Multiple extracted ion electropherograms for the analysis of the RNases and Table S1, a list of all the digested products experimentally observed for tRNAAla GUC (PDF)

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Notes

The authors declare no competing financial interest.

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