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Short communication

## **Optimization of nucleotides dephosphorylation for RNA structural characterization by tandem mass spectrometry hyphenated with separation methods**

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## Abstract

As part of RNA characterization, the identification of post-transcriptional modifications can be performed using hyphenation of separation methods with mass spectrometry. To identify RNA modifications with those methods, a first total digestion followed by a dephosphorylation step are usually required to reduce RNA to nucleosides. Even though effective digestion and dephosphorylation are essential to avoid further complications in analysis and data interpretation, to our knowledge, no standard protocol is yet referenced in the literature. Therefore, the aim of this work is to optimize the dephosphorylation step using a total extract of transfer RNA (tRNA)<sup>1</sup> from *B. taurus* as a model and to determine and fix two protocols, leading to complete dephosphorylation, based on time and bacterial alkaline phosphatase (BAP)<sup>2</sup> consumptions. Capillary electrophoresis-tandem mass spectrometry (CE-MS/MS) was used to estimate the dephosphorylation efficiency of both protocols on many canonical and modified nucleotides. For a timesaving protocol, we established that full dephosphorylation was obtained after a 4-hour incubation at 37 °C with 7.5 U of BAP for 1 µg of tRNA. And for a BAP-saving protocol, we established that full dephosphorylation was obtained 3.0 U of BAP after an overnight incubation at 37 °C. Both protocols are suitable for quantitative analyses as no loss of analytes is expected. Moreover, they can be widely used for all other RNA classes, including messenger RNA or ribosomal RNA.

**Keywords:** ribonucleic acid, modified nucleoside, modified nucleotide, protocols

## Introduction

Over the past few years, RNA studies have shown a growing interest due to the huge potential of RNA in therapeutic strategies such as diagnostic [1], treatment [2] or vaccination [3]. Structural information, including the identification and location of post-transcriptional modifications, is required to characterize RNA. Nowadays, more than 150 modified nucleosides are described in the literature and referenced in databases like Modomics [4]. These modifications are found in all types of RNA and are of great interest due to their

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<sup>1</sup> tRNA : transfer RNA

<sup>2</sup> BAP : bacterial alkaline phosphatase

involvement in the RNA structural stability [5] and many biological functions [6] but also in several diseases [7] as diabetes [8] or cancers [9].

Many techniques have been developed to analyze RNA post-transcriptional modifications, including separative methods such as high-performance liquid chromatography (LC) [8,10–18] or capillary electrophoresis (CE) [19] hyphenated to tandem mass spectrometry (MS/MS). Indeed, on one hand, these separative methods, which have complementary selectivity, enable the characterization of several modified nucleosides including isomers [19]. On the other hand, MS has become a powerful tool for the identification and structural characterization of biomolecules due to its sensitivity and specificity. Using those couplings, the identification of RNA modifications is commonly performed at the nucleosides level, while their localization is determined at the oligonucleotides level. Both, CE and LC, could have been used in this study. However, CE offers the possibility to study RNA at both levels, nucleosides and oligonucleotides, with the same method [19], contrary to LC-MS, while maintaining the advantages of isomeric separation.

In order to digest RNA into nucleosides, the protocols usually described in the literature are based on two steps. The first one is a total digestion with nuclease P1 [10,19] or with a combination of nuclease P1 and phosphodiesterase I [11,20], leading to nucleotides. The second step is a dephosphorylation with bacterial alkaline phosphatase (BAP) [10,11,19–21]. Effective digestion and dephosphorylation are essential to avoid further complications in analysis and data interpretation. In-solution, nuclease P1 seems to be quite efficient with a reported complete digestion of DNA in less than 2 hours [22]. In 1990, Crain *et al* proposed a protocol for both steps leading to full digestion and dephosphorylation [20]. In 2014, Su *et al*, proposed an entire protocol, from the sample preparation to the data interpretation, for the quantitative analysis of RNA modifications by LC-MS. The first digestion step was performed with benzonase and phosphodiesterase I, and the dephosphorylation step with alkaline phosphatase [18]. In 2016, Thüring *et al* published a similar protocol using nuclease P1 instead of benzonase [17]. However, none of them are describing the optimization process of the dephosphorylation step to obtain fully dephosphorylated nucleosides. Moreover, since then, several studies used various protocols [11,19,21]. A kit is also commercialized by New England BioLabs enabling one-step digestion and dephosphorylation of RNA and DNA, however no information on its enzyme composition is available. Therefore,

we consider that there is no standard protocol for the dephosphorylation of nucleotides. Moreover, to the best of our knowledge, analyte recovery rates were never discussed as no step may implies a loss of analytes, and performance studies have not always been carried out for each new protocol, leaving the possibility of partial dephosphorylation. Indeed, most of the studies are using Multiple Reaction Monitoring (MRM) detection [8,11–14,16–18,21,23] as it is really useful to increase sensitivity. However, it is not appropriate for untargeted studies in which nucleotides are remaining in the sample due to poor dephosphorylation or when nucleoside modifications are still unknown.

In this work, we propose to optimize the dephosphorylation step using BAP to fix the conditions of two different protocols based on two classical imperatives: (i) economical with reduced BAP consumption or (ii) fast with a short dephosphorylation time.

## 1. Materials and methods

### 2.1. Chemicals

The chemicals used were of analytical grade or high purity. Water was obtained using a Direct-Q3 UV-R water purification system (Merck Millipore, Amsterdam, The Netherlands). Nuclease P1 from *Penicillium citrinum*, ammonium acetate and zinc chloride (ZnCl<sub>2</sub>) were purchased from Sigma–Aldrich (Saint Louis, MO, U.S.A.). Glacial acetic acid and *Bacterial Alkaline Phosphatase* (BAP) (150 U/μL) from *Escherichia coli* C90 was purchased from ThermoFisher Scientific (Marietta, OH, U.S.A.).

### 2.2. tRNA sample

A total extract of transfer RNA (tRNA) from eukaryotic cytosol of *B. taurus* was used to perform this study. The modifications from *B. taurus* tRNA are numerous and well known, making it a good model to track dephosphorylation performances. All modifications reported in Modomics database [4] for *B. taurus* tRNA total extract are listed in Table 1.

**Table 1.** List of modifications reported in Modomics database [4] for *B. taurus* tRNA total extract

Nucleoside full name	Nucleosides abbreviations	Nucleotides abbreviations
Adenosine	A	pA
Cytidine	C	pC
Guanosine	G	pG
Uridine	U	pU

1-methyladenosine	m <sup>1</sup> A	pm <sup>1</sup> A
3-methylcytidine	m <sup>3</sup> C	pm <sup>3</sup> C
5-methylcytidine	m <sup>5</sup> C	pm <sup>5</sup> C
2'-O-methylcytidine	Cm	pCm
1-methylguanosine	m <sup>1</sup> G	pm <sup>1</sup> G
N2-methylguanosine	m <sup>2</sup> G	pm <sup>2</sup> G
7-methylguanosine	m <sup>7</sup> G	pm <sup>7</sup> G
2'-O-methylguanosine	Gm	pGm
N2,N2,2'-O-trimethylguanosine	m <sup>2</sup> <sub>2</sub> G	pm <sup>2</sup> <sub>2</sub> G
N6-isopentenyladenosine	i <sup>6</sup> A	pi <sup>6</sup> A
3-(3-amino-3-carboxypropyl)uridine	acp <sup>3</sup> U	pacp <sup>3</sup> U
Queuosine	Q	pQ
Galactosyl-queuosine	galQ	pgalQ
Mannosyl-queuosine	manQ	pmanQ
Pseudouridine	Y	pY
Dihydrouridine	D	pD
5-methyluridine	m <sup>5</sup> U	pm <sup>5</sup> U
2'-O-methyluridine	Um	pUm
2'-O-methylpseudouridine	Ym	pYm
5,2'-O-dimethyluridine	m <sup>5</sup> Um	pm <sup>5</sup> Um
N4-acetylcytidine	ac <sup>4</sup> C	pac <sup>4</sup> C
5-methoxycarbonylmethyl-2-thiouridine	mcm <sup>5</sup> s <sup>2</sup> U	pmcm <sup>5</sup> s <sup>2</sup> U
Inosine	I	pI
N6-threonylcarbamoyladenine	t <sup>6</sup> A	pt <sup>6</sup> A
Methoxycarbonylmethyluridine	mcm <sup>5</sup> U	pmcm <sup>5</sup> U
Peroxywybutosine	o <sub>2</sub> yW	po <sub>2</sub> yW
5-formyl-2'-O-methylcytidine	f <sup>5</sup> Cm	pf <sup>5</sup> Cm

### 2.3. Sample preparation

*B. taurus* tRNA was desalted by three successive precipitations with ammonium acetate and ethanol. A single batch of total tRNA extract was digested with nuclease P1 following a previously described protocol [19]. Briefly, P1 10x buffer (2 mM ZnCl<sub>2</sub>, 250 mM ammonium acetate, pH 5.0) and tRNA extract were successively added into deionized water before adding 0.5 U of P1 (previously prepared at 0.5 U/μL in 100 mM ammonium acetate) per μg of tRNA. This single batch of tRNA was incubated at 37 °C for 2 hours, and then used for different dephosphorylation conditions. Nucleotides were dephosphorylated using seven different ratios from 0.15 to 7.5 U of BAP (previously prepared at 1.5 U/μL in 100 mM ammonium acetate) for 1 μg of tRNA and incubated at 37 °C for two periods of time: 4 hours and overnight. The final concentration of tRNA in each sample was 0.1 μg/μL. For each BAP quantity and incubation time combinations, samples were prepared in triplicate and directly analyzed. Analyses of sample triplicates were carried out on different days.

#### 2.4. CE-MS/MS analysis

CE-MS/MS analyses were performed following a previously described method [19]. Briefly, CE was performed on a bare-fused silica capillary (100 cm total length, 30  $\mu\text{m}$  i.d.) filled with 10% acetic acid as background electrolyte. Analyses were performed with 5 nL injections and +30kV separation voltage and 2 psi pressure. A porous tip sheathless interface was used for the CE-MS hyphenation and MS detection was performed in positive mode. MS/MS detection was performed using data-dependent mode (theoretical precursor and product ions used for the identification are available in Supplemental Information in Table S1). To ensure the full dephosphorylation of a maximum of various nucleotides, dephosphorylation efficiencies of all modifications from *B. taurus* tRNA were monitored. However, two modifications reported in Table 1 ( $\text{o}_2\text{yW}$  and  $\text{f}^5\text{Cm}$ ) were withdrawn for the dephosphorylation study due to the lack of signal of corresponding nucleotides or nucleosides in control samples without any dephosphorylation step or with full dephosphorylation. Therefore, the dephosphorylation of 4 canonical and 25 modified nucleotides was monitored (Table 1).

#### 2.5. Verification of P1 digestion step

As some miscleavages of modified nucleosides were previously reported when using only nuclease P1 [20], it was essential to check the performance of the P1 digestion step in our protocols (as P1 is not retrieved before the second incubation, total P1 incubation times are 2 h + 4 h and 2 h + overnight) before making any conclusion. Indeed, it is worth noting that any difference in the digestion efficiency could have an impact on the results obtained for the dephosphorylation step. To ensure that P1 digestion was complete, the ion currents of dinucleotides combining each modification with all four canonical nucleotides were extracted with a mass tolerance of  $\pm 0.02$  Da. Since none of those dinucleotides were detected, we considered that the digestion with nuclease P1 was complete for all studied nucleosides and nucleotides in all samples. As mentioned in the literature, only dinucleotides have been reported after uncomplete digestion with P1 [20]. Consequently, the following study can be focused only on the dephosphorylation step. The ion current of all nucleosides and their corresponding nucleotide species were also extracted with a mass tolerance of  $\pm 0.02$  Da.

## 2.6. Estimation of the dephosphorylation efficiency

The dephosphorylation efficiency was then estimated for each canonical and modified nucleotides based on the following equation:

$$\%_{\text{dephosphorylation}} \approx \frac{Area_{\text{nucleoside}}}{Area_{\text{nucleoside}} + Area_{\text{nucleotide}}} \times 100 \quad (1)$$

$Area_{\text{nucleoside}}$  and  $Area_{\text{nucleotide}}$  are peak areas obtained from extracted ions electropherograms. As nucleosides and nucleotides may have various ionization efficiencies, it is important to note that this dephosphorylation efficiency is only an estimation. However, confidence in full dephosphorylation is obtained when its efficiency is estimated to 100%, which is the main aim of this study.

For post-transcriptional modifications leading to some isomers partially or not separated by CE, the sum of their areas was used and a common dephosphorylation efficiency was calculated. This is the case of  $m^3C$ ,  $m^5C$  and  $Cm$ , as well as  $galQ$  and  $manQ$ ,  $m^1G$  and  $m^2G$ , or  $U$  and  $Y$  (Table S2 in supplemental information).

## 3. Results and discussion

An example of uncomplete dephosphorylation CE-MS/MS analysis is presented in Fig. 1, and the influence of the dephosphorylation efficiency on the analysis is illustrated by the electropherograms in Fig. 2. Full overall dephosphorylation was obtained for 7.5 U of BAP for 1  $\mu\text{g}$  of RNA incubated at 37 °C for 4 hours (Fig. 2B) and for 3.0 U of BAP for 1  $\mu\text{g}$  of RNA incubated overnight at 37 °C (Fig. 2C). Among these protocols enabling full dephosphorylation of nucleotides, the first one with a 4-hour incubation fits with the protocol developed in our laboratory and described by Lechner *et al* [19], and the overnight protocol is a new combination of incubation time and amount of BAP compared to the literature.

The full dephosphorylation enabled the reduction of CE analysis time (Fig. 2B and Fig. 2C) due to late migration of many nucleotides negatively charged. For instance, the migration time of pU was around 27 min (Fig. 2A), thus increasing the analysis time that is around 17 min obtained with full dephosphorylation (Fig. 2B and Fig. 2C). Furthermore, it prevents the potential ion suppression caused by the comigration of some nucleotides with nucleosides.

To illustrate this point, all nucleosides and nucleotides have been annotated in Fig. 1, and canonical nucleosides and nucleotides have been annotated in Fig. 2, particularly showing the peak overlapping of U, D, m<sup>5</sup>U/Um/Ym, m<sup>5</sup>Um, pm<sup>3</sup>C/pm<sup>5</sup>C/pCm, pA<sub>7</sub>, pC, and other species at around 17 min (Fig. 2A). As U and other modified nucleosides are neutral, ionization competition with co-migrating nucleotides can have a huge impact on their detection. Indeed, ion suppression phenomenon can lead to a loss of sensitivity, particularly for low concentrated compounds, which is often the case of modified nucleosides. In turn, low peak intensities can lead to a failure of the automatic MS/MS fragmentation process in data-dependent mode resulting in a loss of characterization for some nucleosides. Finally, with uncompleted dephosphorylation, the occurrence of each modification is split between both nucleoside and nucleotide species (Fig. 1 and Fig. 2A). Full dephosphorylation is therefore essential for quantification studies avoiding the underestimation or overestimation of RNA modifications. Our protocols should be well suited for relative quantitation analysis as well as absolute quantitation studies with the use of internal standards. Indeed, no step in the sample treatment that could cause analyte loss, such as post-digestion desalting, is required.

Fig. 3 represents the dephosphorylation efficiency for 29 nucleosides for 4-hour and overnight incubations carried out with seven increasing quantities of BAP (0.15 to 7.5 U) for 1 µg of tRNA. The results for each condition correspond to average dephosphorylation efficiencies obtained from triplicates. Full dephosphorylation was considered for efficiencies equal or higher than 98 %. All standard errors were below ± 19% for uncompleted dephosphorylations and below ± 3% for full dephosphorylations (Tables S3 and S4 in supplemental information). As analyses of sample triplicates were carried out on different days, this shows that the estimated day-to-day variability is relatively low.

For a given incubation time, the minimum quantity of BAP needed to obtain full dephosphorylation is not the same for all modifications. For instance, many modifications including m<sup>1</sup>G, m<sup>2</sup>G, Gm, acp<sup>3</sup>U and ac<sup>4</sup>C are fully dephosphorylated with 4.5 U of BAP after a 4-hour incubation at 37°C, whereas dephosphorylation efficiencies of m<sup>7</sup>G, Q, manQ and galQ do not exceed 81 % for the same BAP quantity and incubation time (Fig. 23A).

Even if small differences in the dephosphorylation efficiencies could have been expected between large and distinctive modifications, the huge gap in the dephosphorylation

efficiencies is more surprising between small-sized isomeric modifications with close structures. For instance, no conclusion can be made regarding a potential difference in dephosphorylation efficiencies between  $m^1G$  and  $m^2G$  as they are not separated by the CE. However, the sum of  $m^1G$  and  $m^2G$  (annotated  $m^1G/m^2G$ ) is already more than half dephosphorylated with the softest condition (0.15 U of BAP for 1  $\mu$ g of tRNA incubated for 4 hours at 37 °C) while  $m^7G$  and Gm are barely dephosphorylated (Fig. 3A). The proportion shift of  $pm^7G$ ,  $pm^1G/pm^2G$  and pGm between 0.15 U and 1.5 U of BAP incubated for 4 hours at 37 °C, as well as their chemical structures, are illustrated in Fig. 4. Even if  $pm^1G/pm^2G$  were after a 4-hour incubation at 37°C with 0.15 U of BAP per  $\mu$ g of RNA (Fig. 4A),  $pm^7G$  was the only isomer not fully dephosphorylated with 4.5 U of BAP per  $\mu$ g of RNA (Fig. 4E). This proportion shift indicates that  $m^7G$  has the lowest dephosphorylation rate of the four methylated guanosine isomers.

After digestion with nuclease P1, nucleotides with 3'-OH and 5'-NMP ends are obtained [24]. Therefore, the bad dephosphorylation of  $m^7G$  compared to  $m^1G$ / ~~$m^2G$~~  and Gm is quite surprising as the residual phosphate group is linked to the ribose and not to the modified base. Due to the positions of methylation in Gm and  $m^7G$ , based on the reaction mechanism of BAP described in Yang and coworker research [25], we rather expected Gm than the other isomers to be the most difficult to dephosphorylate or at least to have similar dephosphorylation efficiencies because of the light steric hindrance of the methylations. Besides these inhomogeneous dephosphorylation rates, it is also worth noting that the first nucleoside at the 5'-end of each tRNA sequence does not possess a phosphate group and moreover is usually not modified. Thus, there is already a small amount of unmodified nucleosides in the sample even without any dephosphorylation step. This could correlate to the higher dephosphorylation efficiency of G compared to Gm and  $m^7G$  displayed for the softest condition (0.15 U of BAP for 1  $\mu$ g of tRNA incubated for 4 hours at 37 °C) in Fig. 23A. However, regarding each modification, dephosphorylation efficiencies higher than 98% were obtained for 7.5 U of BAP for 1  $\mu$ g of RNA incubated at 37 °C for 4 hours and for 3.0 U of BAP for 1  $\mu$ g of RNA incubated overnight at 37 °C.

## 4. Conclusion

The most remarkable feature of this study is that two equivalent protocols can be established for the nucleotides dephosphorylation in routine for CE-MS/MS but also LC-MS/MS analysis purposes. According to individual needs:

(i) the first one is quicker but the amount of enzyme matters (7.5 U of BAP for 1 µg of RNA incubated at 37 °C for 4 hours),

(ii) the second one is longer but more economical in enzyme quantity (3.0 U of BAP for 1 µg of RNA incubated overnight at 37 °C).

## Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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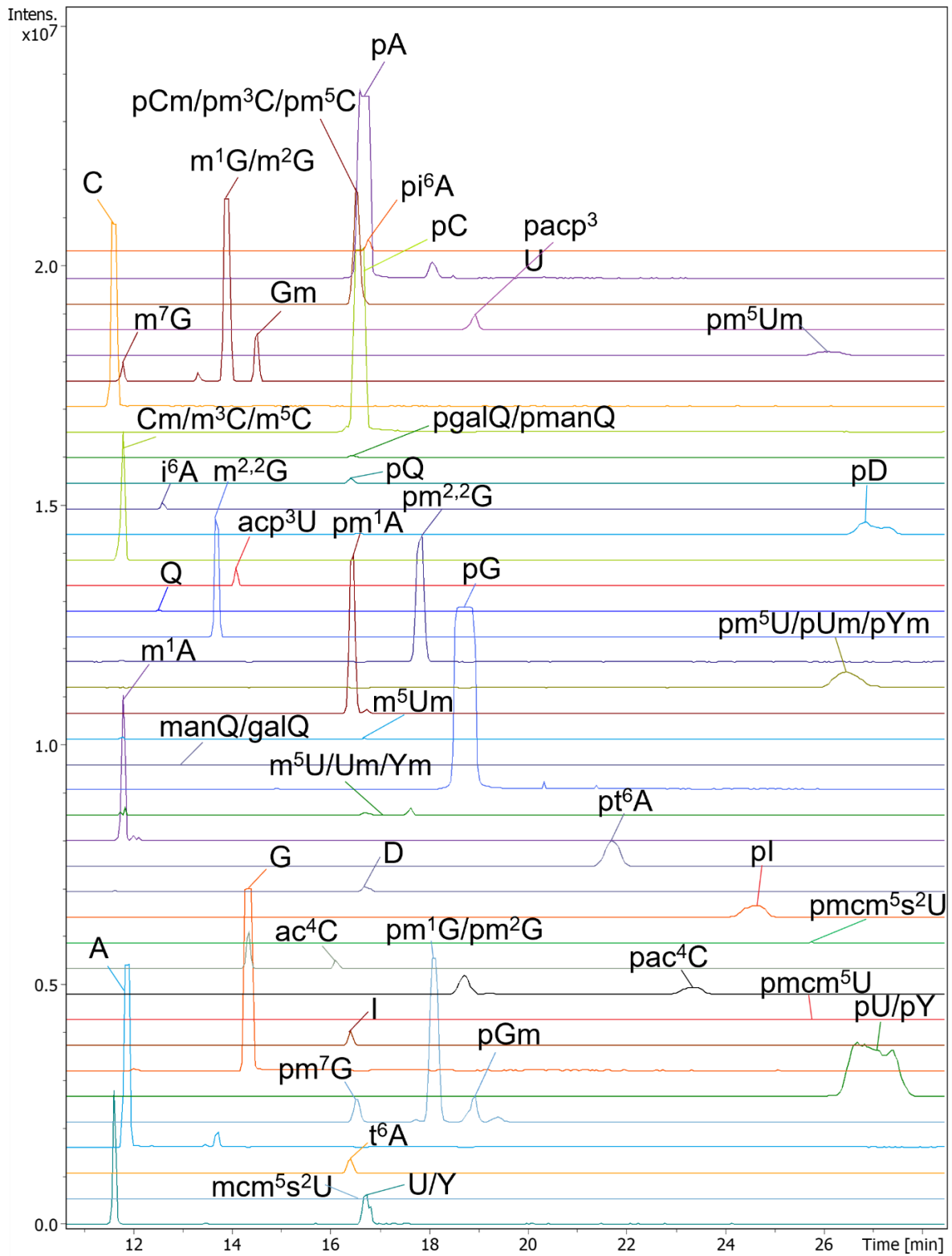
## References

- [1] D. Lu, T. Thum, RNA-based diagnostic and therapeutic strategies for cardiovascular disease, *Nat Rev Cardiol.* 16 (2019) 661–674. <https://doi.org/10.1038/s41569-019-0218-x>.
- [2] D. Adams, A. Gonzalez-Duarte, W.D. O’Riordan, C.-C. Yang, M. Ueda, A.V. Kristen, I. Tournev, H.H. Schmidt, T. Coelho, J.L. Berk, K.-P. Lin, G. Vita, S. Attarian, V. Planté-Bordeneuve, M.M. Mezei, J.M. Campistol, J. Buades, T.H. Brannagan, B.J. Kim, J. Oh, Y. Parman, Y. Sekijima, P.N. Hawkins, S.D. Solomon, M. Polydefkis, P.J. Dyck, P.J. Gandhi, S. Goyal, J. Chen, A.L. Strahs, S.V. Nochur, M.T. Sweetser, P.P. Garg, A.K. Vaishnav, J.A. Gollob, O.B. Suhr, Patisiran, an RNAi Therapeutic, for Hereditary Transthyretin Amyloidosis, *N Engl J Med.* 379 (2018) 11–21. <https://doi.org/10.1056/NEJMoa1716153>.

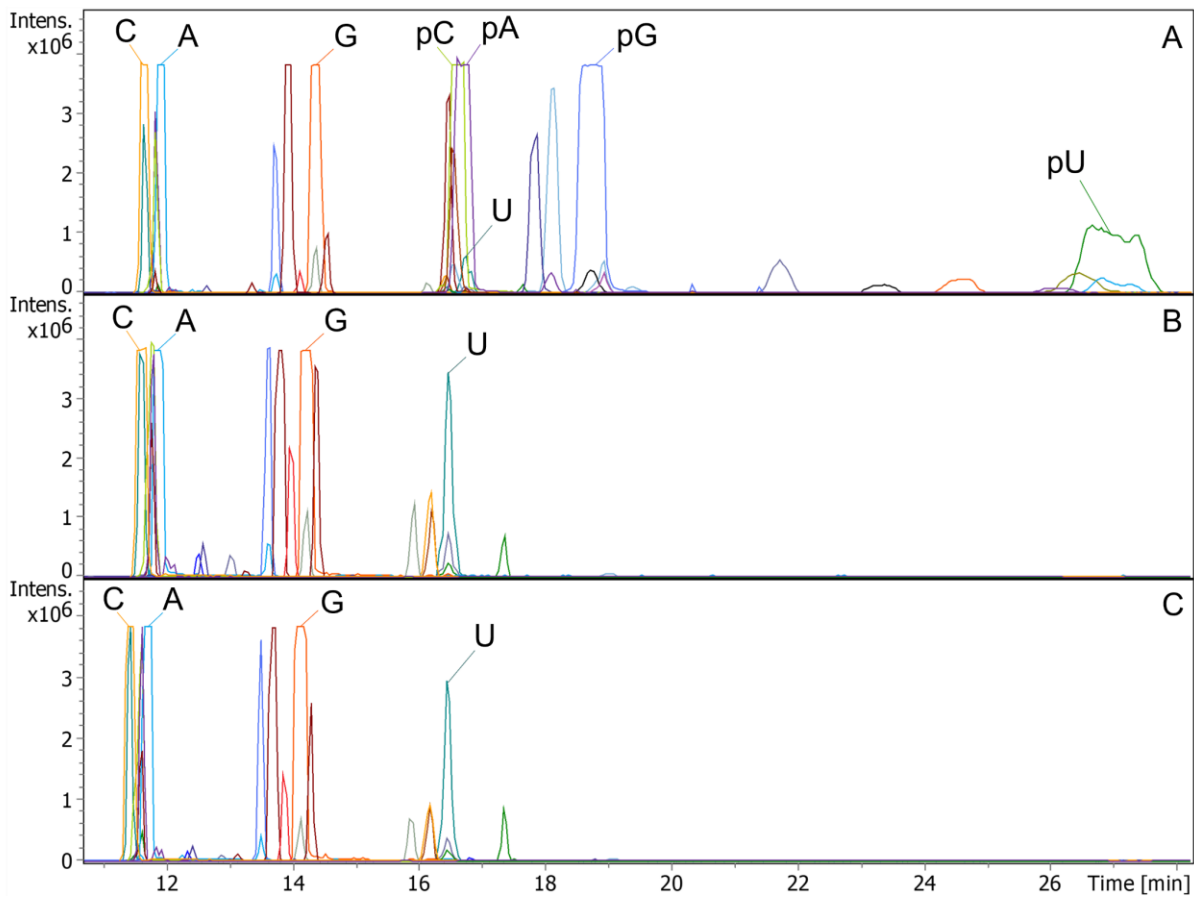
- [3] N. Pardi, M.J. Hogan, F.W. Porter, D. Weissman, mRNA vaccines — a new era in vaccinology, *Nat Rev Drug Discov.* 17 (2018) 261–279. <https://doi.org/10.1038/nrd.2017.243>.
- [4] P. Boccaletto, F. Stefaniak, A. Ray, A. Cappannini, S. Mukherjee, E. Purta, M. Kurkowska, N. Shirvanizadeh, E. Destefanis, P. Groza, G. Avşar, A. Romitelli, P. Pir, E. Dassi, S.G. Conticello, F. Aguilo, J.M. Bujnicki, MODOMICS: a database of RNA modification pathways. 2021 update, *Nucleic Acids Research.* 50 (2022) D231–D235. <https://doi.org/10.1093/nar/gkab1083>.
- [5] S.H. Boo, Y.K. Kim, The emerging role of RNA modifications in the regulation of mRNA stability, *Exp Mol Med.* 52 (2020) 400–408. <https://doi.org/10.1038/s12276-020-0407-z>.
- [6] P.F. Agris, The Importance of Being Modified: Roles of Modified Nucleosides and Mg<sup>2+</sup> in RNA Structure and Function, in: W.E. Cohn, K. Moldave (Eds.), *Progress in Nucleic Acid Research and Molecular Biology*, Academic Press, 1996: pp. 79–129. [https://doi.org/10.1016/S0079-6603\(08\)60143-9](https://doi.org/10.1016/S0079-6603(08)60143-9).
- [7] M.T. Bohnsack, K.E. Sloan, The mitochondrial epitranscriptome: the roles of RNA modifications in mitochondrial translation and human disease, *Cell Mol Life Sci.* 75 (2018) 241–260. <https://doi.org/10.1007/s00018-017-2598-6>.
- [8] M. Yan, Y. Wang, Y. Hu, Y. Feng, C. Dai, J. Wu, D. Wu, F. Zhang, Q. Zhai, A High-Throughput Quantitative Approach Reveals More Small RNA Modifications in Mouse Liver and Their Correlation with Diabetes, *Anal. Chem.* 85 (2013) 12173–12181. <https://doi.org/10.1021/ac4036026>.
- [9] A. Amalric, A. Bastide, A. Attina, A. Choquet, J. Vialaret, S. Lehmann, A. David, C. Hirtz, Quantifying RNA modifications by mass spectrometry: a novel source of biomarkers in oncology, *Crit. Rev. Clin. Lab. Sci.* 0 (2021) 1–18. <https://doi.org/10.1080/10408363.2021.1958743>.
- [10] Y. Sakaguchi, K. Miyauchi, B. Kang, T. Suzuki, Chapter Two - Nucleoside Analysis by Hydrophilic Interaction Liquid Chromatography Coupled with Mass Spectrometry, in: C. He (Ed.), *Methods in Enzymology*, Academic Press, 2015: pp. 19–28. <https://doi.org/10.1016/bs.mie.2015.03.015>.
- [11] P. Wolff, C. Villette, J. Zumsteg, D. Heintz, L. Antoine, B. Chane-Woon-Ming, L. Droogmans, H. Grosjean, E. Westhof, Comparative patterns of modified nucleotides in individual tRNA species from a mesophilic and two thermophilic archaea, *RNA.* 26 (2020) 1957–1975. <https://doi.org/10.1261/rna.077537.120>.
- [12] R. Liu, F. Zhao, J. Wei, P. Yu, J. Zhang, Y. Wang, Z. Li, J. Zhang, X. Zhang, X. Tian, Determination of five nucleosides by LC-MS/MS and the application of the method to quantify N6-methyladenosine level in liver messenger ribonucleic acid of an acetaminophen-induced hepatotoxicity mouse model, *J. Sep. Sci.* 42 (2019) 2668–2678. <https://doi.org/10.1002/jssc.201900041>.
- [13] E. Raćkowska, B. Bobrowska-Korczak, J. Giebułtowski, Development and validation of a rapid LC-MS/MS method for determination of methylated nucleosides and nucleobases in urine, *J. Chromatogr. B.* 1128 (2019) 121775. <https://doi.org/10.1016/j.jchromb.2019.121775>.
- [14] W.-Y. Lo, L.-B. Jeng, C.-C. Lai, F.-J. Tsai, C.-T. Lin, W.T.-L. Chen, Urinary cytidine as an adjunct biomarker to improve the diagnostic ratio for gastric cancer in Taiwanese patients, *Clin. Chim. Acta.* 428 (2014) 57–62. <https://doi.org/10.1016/j.cca.2013.10.008>.

- [15] F. Chen, F. Zhang, N. Yang, X. Liu, Simultaneous Determination of 10 Nucleosides and Nucleobases in *Antrodia camphorata* Using QTRAP LC–MS/MS, *J. Chromatogr. Sci.* 52 (2014) 852–861. <https://doi.org/10.1093/chromsci/bmt128>.
- [16] Y.-J. Feng, X.-J. You, J.-H. Ding, Y.-F. Zhang, B.-F. Yuan, Y.-Q. Feng, Identification of Inosine and 2'-O-Methylinosine Modifications in Yeast Messenger RNA by Liquid Chromatography–Tandem Mass Spectrometry Analysis, *Anal. Chem.* 94 (2022) 4747–4755. <https://doi.org/10.1021/acs.analchem.1c05292>.
- [17] K. Thüring, K. Schmid, P. Keller, M. Helm, Analysis of RNA modifications by liquid chromatography–tandem mass spectrometry, *Methods.* 107 (2016) 48–56. <https://doi.org/10.1016/j.ymeth.2016.03.019>.
- [18] D. Su, C.T.Y. Chan, C. Gu, K.S. Lim, Y.H. Chionh, M.E. McBee, B.S. Russell, I.R. Babu, T.J. Begley, P.C. Dedon, Quantitative analysis of ribonucleoside modifications in tRNA by HPLC-coupled mass spectrometry, *Nat Protoc.* 9 (2014) 828–841. <https://doi.org/10.1038/nprot.2014.047>.
- [19] A. Lechner, P. Wolff, E. Leize-Wagner, Y.-N. François, Characterization of Post-Transcriptional RNA Modifications by Sheathless Capillary Electrophoresis–High Resolution Mass Spectrometry, *Anal. Chem.* 92 (2020) 7363–7370. <https://doi.org/10.1021/acs.analchem.0c01345>.
- [20] P.F. Crain, Preparation and enzymatic hydrolysis of DNA and RNA for mass spectrometry, in: *Methods in Enzymology*, Academic Press, 1990: pp. 782–790. [https://doi.org/10.1016/0076-6879\(90\)93450-Y](https://doi.org/10.1016/0076-6879(90)93450-Y).
- [21] J. Wang, H. Dong, Y.H. Chionh, M.E. McBee, S. Sirirungruang, R.P. Cunningham, P.-Y. Shi, P.C. Dedon, The role of sequence context, nucleotide pool balance and stress in 2'-deoxynucleotide misincorporation in viral, bacterial and mammalian RNA, *Nucleic Acids Research.* 44 (2016) 8962–8975. <https://doi.org/10.1093/nar/gkw572>.
- [22] Y. Ma, B. Chen, D. Zhang, Quantitation of DNA by nuclease P1 digestion and UPLC–MS/MS to assess binding efficiency of pyrrolobenzodiazepine, *J. Pharm. Anal.* 10 (2020) 247–252. <https://doi.org/10.1016/j.jpha.2020.05.011>.
- [23] P. Gątarek, J. Kałużna-Czaplińska, M. Pawełczyk, K. Jastrzębski, J. Giebułtowicz, A. Głąbiński, B. Bobrowska-Korczak, LC-MS/MS Determination of Modified Nucleosides in The Urine of Parkinson's Disease and Parkinsonian Syndromes Patients, *Molecules.* 25 (2020) 4959. <https://doi.org/10.3390/molecules25214959>.
- [24] W. Yang, Nucleases: diversity of structure, function and mechanism, *Q. Rev. Biophys.* 44 (2011) 1–93. <https://doi.org/10.1017/S0033583510000181>.
- [25] K. Yang, W.W. Metcalf, A new activity for an old enzyme: *Escherichia coli* bacterial alkaline phosphatase is a phosphite-dependent hydrogenase, *PNAS.* 101 (2004) 7919–7924. <https://doi.org/10.1073/pnas.0400664101>.

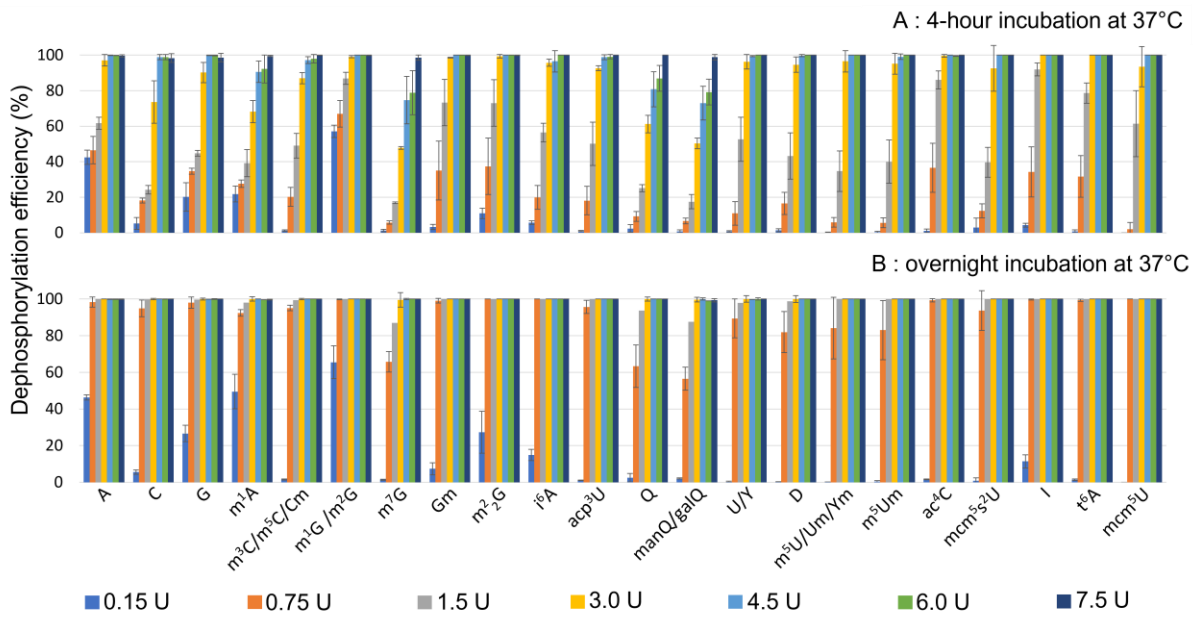
## Captions



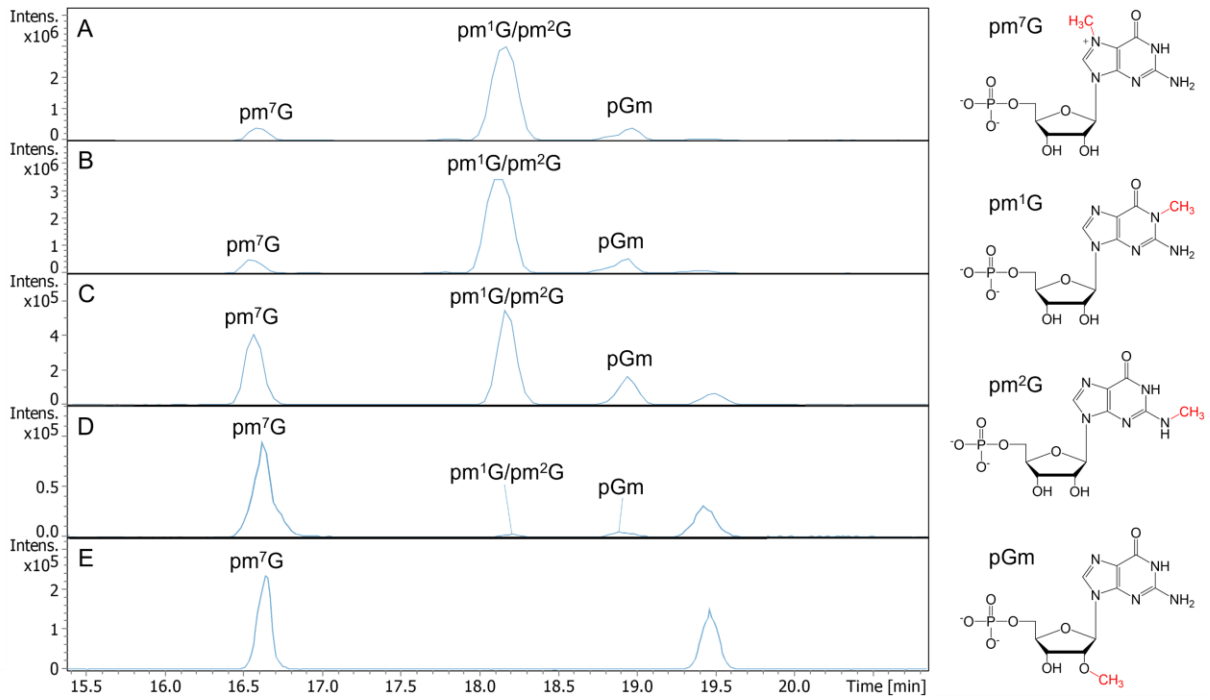
**Fig. 1.** Multiple extracted ion electropherograms for the analysis of total tRNA extract from *B. taurus* after digestion with nuclease P1 and dephosphorylation after a 4-hour incubation at 37°C with 0.75 U of BAP per  $\mu g$  of RNA



**Fig. 2.** Multiple extracted ion electropherograms for the analysis of total tRNA extract from *B. taurus* after digestion with nuclease P1 and dephosphorylation with (A) 0.75 U of BAP for 1  $\mu$ g of RNA incubated for 4 hours at 37 °C, (B) 7.5 U of BAP for 1  $\mu$ g of RNA incubated for 4 hours at 37 °C and (C) 3.0 U of BAP for 1  $\mu$ g of RNA incubated for 4 hours at 37 °C. All 4 canonical and 25 modified nucleotides and nucleosides were extracted. Full annotation of nucleoside and nucleotides of Fig. 2A is available in Fig. 1.



**Fig. 3.** Estimated dephosphorylation efficiencies for 29 nucleotides after (A) 4-hour incubation and (B) overnight incubation at 37°C with seven quantities from 0.15 to 7.5 U of BAP for 1 µg of RNA. Standard errors were obtained from sample triplicates. More precise data are available in Supplemental Information (Tables S2 and S3).



**Fig. 4.** Extracted ion electropherogram ( $m/z$  378.08) for the analysis total tRNA extract from *B. taurus* after digestion with nuclease P1 and dephosphorylation after a 4-hour incubation at 37°C with (A) 0.15 U of BAP per  $\mu\text{g}$  of RNA, (B) 0.75 U of BAP per  $\mu\text{g}$  of RNA, (C) 1.5 U of BAP per  $\mu\text{g}$  of RNA, (D) 3.0 U of BAP per  $\mu\text{g}$  of RNA and (E) 4.5 U of BAP per  $\mu\text{g}$  of RNA. (F) Structures of all four isomers of methylated monophosphate guanosine.