Application note | 002601

Thermo Fisher SCIENTIFIC

Biopharma

Confident intact mass analysis of the tRNA isodecoders of phenylalanine by UHPLC-HRAM-MS and BioPharma Finder software

Authors

Robert L. Ross[1], Keeley Murphy[1], Roberto Gamez[2], Paul R. Gazis[2], Jennifer Sutton[2], Min Du[1]

[1]Thermo Fisher Scientific, Lexington, MA, USA

[2]Thermo Fisher Scientific, San Jose, CA, USA

Keywords

Oligonucleotide, chemical modifications, ribonucleic acid (RNA), transfer RNA (tRNA), high-resolution accurate-mass (HRAM), ion-pair reversed-phase liquid chromatography (IP-RP-LC), ultra high performance liquid chromatography (UHPLC), UHPLC-HRAM-MS, intact mass analysis, Vanquish Horizon UHPLC, DNAPac RP column, Orbitrap Ascend Tribrid mass spectrometer, BioPharma Finder software

Application benefits

- Confident and sensitive identification of biological transfer ribonucleic acid (tRNA) and their modifications is achieved using high-resolution accurate-mass (HRAM) mass spectrometry.
- Ion pair reversed-phase liquid chromatography (IP-RP-LC) using the polymer-based Thermo Scientific™ DNAPac™ RP column with dibutylamine increases the electrospray signal while simultaneously minimizing Na^+ adducts.
- The fully biocompatible ultra high performance liquid chromatography (UHPLC) platform coupled with the Thermo Scientific™ Orbitrap™ HRAM mass spectrometer provides high quality mass spectra of intact species with baseline resolved isotope peak patterns.
- High quality Orbitrap HRAM MS spectra yield mass accuracy less than 5 ppm for large tRNA and its biological variants containing post-transcriptional modifications, ensuring the confident identification even for low abundance species (down to 3% abundance).
- Thermo Scientific™ BioPharma Finder™ software provides an easy to use and flexible intact mass analysis workflow for fast data processing, annotation, and review.

thermo scientific

Goal

Demonstrate the intact mass analysis of a biological tRNA using the Thermo Scientific™ Orbitrap™ Ascend Tribrid™ mass spectrometer with BioPharma Finder 5.2 software and the polymer-based DNAPac RP column

Introduction

Over 140 chemical modifications to the canonical base and/or sugar of ribonucleic acid (RNA) have been reported.[1] By far the greatest density of modification is found in tRNA. Modification in the anticodon loop has been shown to assist in codon recognition and reading frame stability during mRNA translation. Other modifications, such as methylation, have been shown to help maintain the three-dimensional structure of the tRNA or to act as determinates for RNA-protein complexation. Due to the size of tRNA, enzymatic digestion followed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) remains the gold standard in analytical technology for RNA modification identification and characterization. A challenge with enzymatic digestion is the resulting complexity of the sample. Even with chromatographic separation, mapping an oligonucleotide to its sequence context can be difficult. Should the tRNA sequence be known, then global modification status can be determined and monitored through intact mass analysis with accuracies of ~5 ppm. Furthermore, this approach is fast and requires no sample preparation. Here, using ultra-high-performance liquid chromatography - high-resolution accurate-mass mass spectrometry (UHPLC-HRAM-MS) with BioPharma Finder software we show the ability to identify the tRNA isodecoders of phenylalanine ($tRNA^{PHE}$) through intact mass analysis.

Experimental

Reagents, consumables, and lab equipment

- Ribonucleic acid, transfer, phenylalanine specific from brewer's yeast, Sigma-Aldrich (P/N R4018)
- Thermo Scientific™ UHPLC-MS water (P/N W81)
- Thermo Scientific™ UHPLC-MS acetonitrile (P/N A9554)
- Invitrogen™ Nuclease-free water (not DEPC-treated) (P/N 9938)
- Thermo Scientific™ F1-ClipTip™ variable volume single channel pipettes (P/N 4641210N)
- Invitrogen™ Nonstick, RNase-free microfuge tubes, 1.5 mL (P/N AM12450)
- Thermo Scientific™ Pierce™ FlexMix™ calibration solution (P/N A39239)
- Thermo Scientific™ Savant™ SpeedVac™ DNA 130 integrated vacuum concentrator system (P/N DNA130-115)
- Thermo Scientific™ NanoDrop™ One/OneC microvolume UV-Vis spectrophotometer (P/N ND-ONEC-W)
- Thermo Scientific™ 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP), 99%, for analysis, (P/N A12747.22)
- Thermo Scientific™ Di-*n*-butylamine, 98+%, (P/N A11671.AP)

Instrumentation

For all experiments, the Thermo Scientific™ Vanquish™ Horizon UHPLC system was used, consisting of:

- Thermo Scientific™ Vanquish™ System Base F/H (VF-S01-A-02)
- Thermo Scientific™ Vanquish™ Binary Pump H (P/N VH-P10-A-02)
- Thermo Scientific™ Vanquish™ Split Sampler HT (P/N VH-A10-A-02)
- Thermo Scientific™ Vanquish™ Column Compartment H (P/N VH-C10-A-02)
- Thermo Scientific™ Viper™ MS Connection Kit for Vanquish™ LC systems (P/N 6720.0405)

Ion-pair reversed-phase liquid chromatography (IP-RP LC)

Liquid chromatography was performed using a Vanquish Horizon UHPLC system and a Thermo Scientific™ DNAPac™ RP column (4 µm, 2.1 × 100 mm, P/N 088923) column. The autosampler was held at 10 °C while the column was maintained at 75 °C with the column oven thermostat mode set to Still Air. The LC gradient used in this study is shown in Table 1.

Mobile phase A was 25 mM HFIP, 15 mM dibutylamine in H_2O. Mobile phase B was 100% ACN. The flow rate was 0.400 mL/min.

Table 1. UHPLC gradient conditions

Time	%B
0.000	10
0.500	10
4.000	25
6.000	27
7.000	40
7.010	90
7.500	90
7.510	10
12.000	10

Mass spectrometry

The Thermo Scientific™ Orbitrap™ Ascend Tribrid™ mass spectrometer (P/N FSN06-10000) was operated with Thermo Scientific™ Xcalibur™ 4.6 software and controlled by Thermo Scientific™ Orbitrap™ Tribrid™ instrument control software 4.0 SP1 (ICSW). Instrument calibration was performed using Thermo Scientific™ Pierce™ FlexMix™ calibration solution. Data acquisition was performed in negative ion mode.

The MS method was built in the method editor using the standard MS template provided with the Orbitrap Ascend Tribrid instrument control software and then modified accordingly. Table 2 lists the scan parameters used in these acquisitions. Thermo Scientific™ OptaMax™ NG ion source settings were default for the flow rate (400 µL/min) used in the experiment.

Table 2. MS parameters used in the analysis

Global settings	Value
Application mode	Intact protein
Pressure mode	Low pressure
Default charge state	10
Advanced peak determination	TRUE
Full scan	**Value**
Orbitrap resolution	240,000
Scan range (*m/z*)	800–3,000
RF lens (%)	70
AGC target	Custom
Normalized AGC target (%)	200
Maximum injection time mode	Custom
Maximum injection time (ms)	300
Microscans	2
Data type	Profile
Polarity	Negative

Software

- Xcalibur 4.6 software
- Thermo Scientific™ Freestyle™ 1.8.2 software
- BioPharma Finder 5.2 software (P/N OPTON-30988)

tRNA intact mass analysis in BioPharma Finder software

To match the intact mass of the observed signal against the theoretical mass, modifications of the canonicals must be created and incorporated into the sequence. The sequence of the yeast tRNA^{PHE} isodecoder and its minor variant, an A to G substitution at position 67 along with its corresponding complement at position 6, was published by Keith in 1987,[2] which showed 11 positions within the tRNA^{PHE} containing a modification (Figure 1).

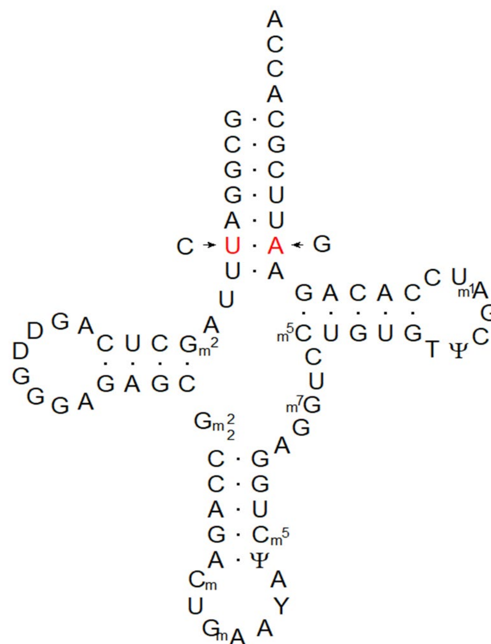

Figure 1. Secondary structure of tRNA^{PHE} showing position of post-transcriptional modifications. Red bases show nucleotide substitution in the minor variant of tRNA^{PHE}. Structure adapted from Keith using RnaViz. 2.0.

As in previous mRNA analyses utilizing BioPharma Finder software,[3,4] the Sequence Manager is used to create and modify the RNA sequence (Figure 2). Other than the standard base or ribose sugar modification, tRNA^{PHE} contains pseudouridine (Ψ), a mass silent uridine analog. The tRNA^{PHE} also contains the modification N,N-dimethylguanosine (m2,2G) as well as the hypermodified tricyclic guanosine modification, wybutosine (yW). Using the chemical drawing software MarvinSketch (chemaxon.com/marvin), each modified nucleoside is created and its chemical formula calculated. Next, the *Building Block and Variable Modification Editor* is opened and from the Subunit dropdown, "Base" is selected. The modification is then named, and its formula, calculated from its molecular drawing, is then added to automatically populate the monoisotopic and average mass fields. Figure 2 gives an example of inputting the nucleobase modification N2,N2-dimethylguanosine (m2,2G), a modification found on the guanosine at position 10 in the tRNA^{PHE} sequence. This process is repeated to create all the modifications within the tRNA^{PHE}.

Figure 2. Building Block and Variable Modification Editor is used to create modifications within the sequence, added as a molecular formula that can be generated through a chemical drawing.

After creation of the modifications, the Edit Sequence panel is accessed. It is from this panel that any nucleotide in the sequence can be modified, using either the default modifications in the dropdown menus, or the user-created modifications generated in the Building Block and Variable Modification Editor. Any change to the sequence in the edit sequence panel is reflected in the Oligo Sequence Map. For example, addition of the m2,2G base generated in the Building Block editor is chosen as the base from the dropdown menu in the Edit Sequence panel, changing the guanosine to the m2,2G modification (Figure 3A). When the base substitution is applied, the change is reflected as an addition of an uppercase (Z) at position 10 in the tRNA sequence (Figure 3B) The uppercase (Z) is a user-defined symbol chosen when the modification is created. Once all the modifications are entered and applied, the monoisotopic mass is displayed in the Sequence Information pane (Figure 3C).

After all sequences are created, the next step is to create the analysis method in the BioPharma Finder Intact Mass Analysis workflow. The parameters used for this analysis are listed in Table 3.

Figure 3. BioPharma Finder Sequence Manager panels showing the incorporation of the custom modification m2,2G into the tRNA sequence. (A) The Edit Sequence pane, here the modification is incorporated into the sequence through the drop-down menu available for the base. (B) The Oligo Sequence Map automatically updates showing position within the sequence. (C) The Sequence Information panel updates the monoisotopic mass reflecting all modification.

Table 3. BioPharma Finder Intact Mass Analysis parameters

Main parameters (Xtract algorithm)	Value
Output mass range (Da)	22,000–26,000
S/N threshold	3
Rel. abundance threshold (%)	4
Charge range	5–50
Min. num. detected charge	3
Isotope table	Nucleotide
Advanced parameters (Xtract algorithm)	**Value**
Calculate XIC	FALSE
Fit factor (%)	80
Remainder threshold (%)	25
Consider overlaps	TRUE
Raw file specific	24,000
Negative charge	TRUE
Charge carrier	H
Minimum intensity	1
Expected intensity error	3

Results and discussion

UHPLC-HRAM-MS of tRNA^{PHE}

The advantage of using dibutylamine in this study as the ion pairing reagent for larger oligonucleotide analysis is the increased hydrophobicity of the nucleic acids that the amine imparts. A more hydrophobic biopolymer can present a cleaner spectrum due to rapid ejection from the electrosprayed droplet.[5,6] Desorbing into the gas phase before the droplet fully evaporates enhances the signal as less adduction of residual Na^+ and K^+ from the mobile phase happens.[7] The recently introduced Orbitrap Ascend Tribrid mass spectrometer is equipped with improved Tribrid architecture and enhanced vacuum technology that improves the accumulation and transmission of high molecular weight ions to the Orbitrap mass analyzer.

For this analysis, a scan range of 800–3,000 *m/z* was used due to the broad charge state envelope presented by the electrosprayed tRNA^{PHE}. Intact mass measurement of tRNA^{PHE} shows a charge state distribution between -9 to -26 with the most abundant charge states between -15 and -19 (Figure 4A). For each charge state, several features were observed (Figure 4B). Deconvolution of the spectra from the main chromatographic peak returns a monoisotopic mass of 24,610.532 Da. Two peaks at 30% relative abundance flanking the most abundant peak had deconvoluted masses at 24,626.526 and 24,596.547, a difference of +16.076 and -13.903 Da from the most abundant peak's mass.

Transfer RNA is known to have a 5' phosphate due to RNase P cleavage post transcription by pol III.[8] In yeast, the phenylalanine isodecoder as well as its minor variant are known to have an adenosine truncation at the 3' terminus.[9,10] Using the verified sequences of the two published isodecoders incorporating a 5' phosphate as well as the 3' adenosine truncation, returns theoretical monoisotopic masses of 24,610.456 and 24,625.466 Da with the major tRNA variant having a mass delta of ~3 ppm.

To identify other species from the list of deconvoluted masses, we constructed a series of masses as a reference to match against the deconvolution results. A truncation series of monoisotopic intact masses for the A67 and G67 mature sequences were created with the largest mass being the full charged sequence (3' terminal CpCpA carrying the amino acid) as well as the 5' terminal phosphate (Table 4). Each sequential mass removes either the base or the phosphate to generate a mass list of truncated sequences. Comparing the calculated values against the deconvoluted results, we identify both variants of the tRNA with the most abundant (100% relative abundance) being the A67 variant having the CpC truncation. We further identify both variants having the expected 3' terminus CpCpA, uncharged, at a relative abundance of ~18% for the major variant A67 and the minor G67 variant at ~5%.

Finally, the unidentified deconvoluted mass of 24596.547 has a delta mass of -13.903 Da from the identified main variant. This mass shift is associated with a loss of a single methyl group. The most labile methylation for tRNA^{PHE} is on the side chain of the hyper-modification wybutosine. Extensive characterization of the yW biosynthetic pathway has previously been reported by Noma, et. al,[11] with the last step in the enzymatic pathway a carboxymethylation of the yW-58 intermediate to yW. A tRNA sequence was created in Sequence Manager using the A67 sequence yet substituting a demethylated wybutosine nucleobase at position 37. Reprocessing the data with the demethylated nucleobase resulted in an identification of the deconvoluted mass 24,596.547 with a delta mass below 5 ppm. Tentative identification of the yW-14 containing sequence resulted in characterization of all deconvoluted signals identified and annotated, shown in Figure 4D with mass deviation, abundance, and charge state tabulated in Table 5.

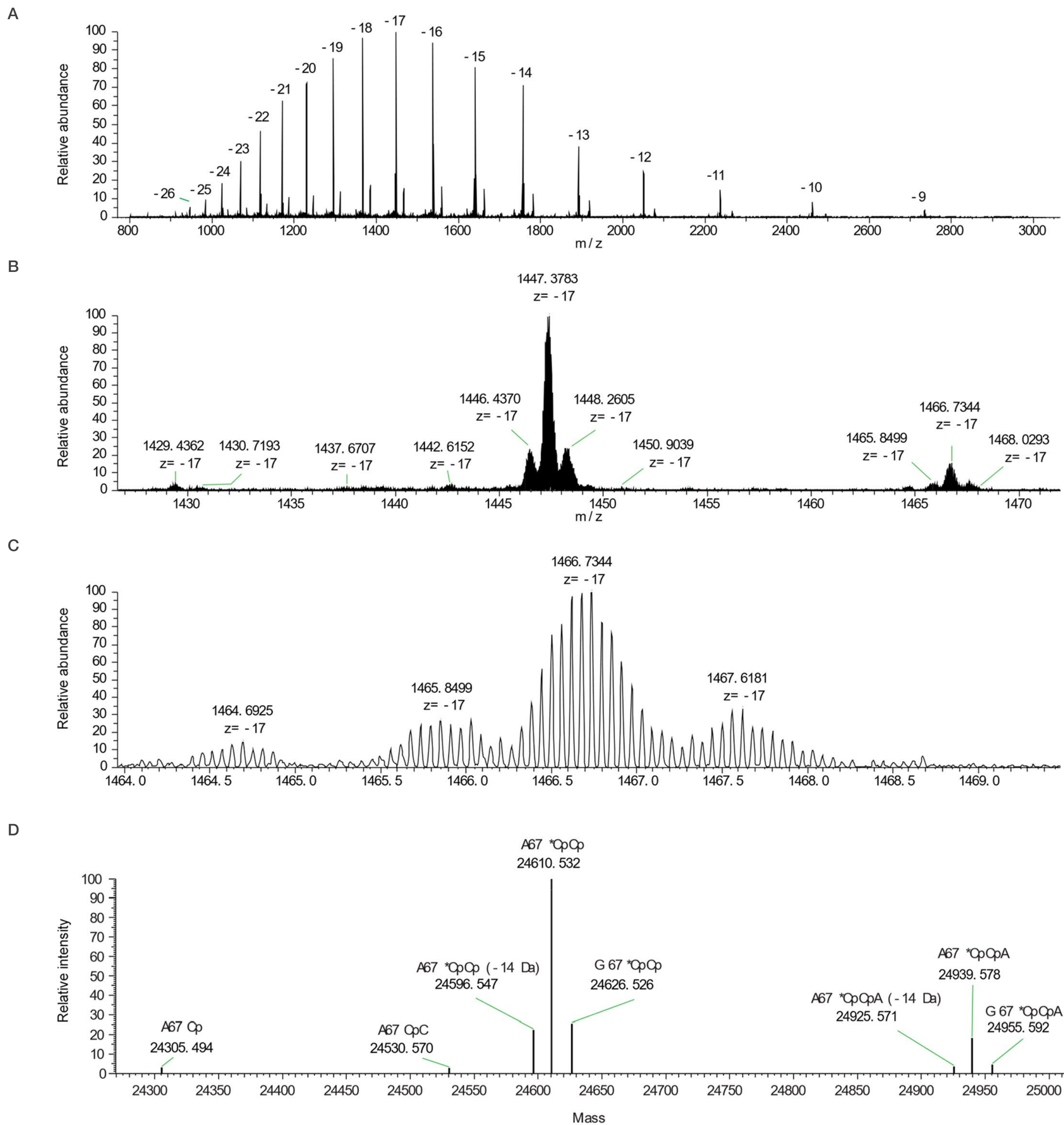

Figure 4. Mass spectrum of a commercially available tRNA^{PHE} standard. (A) Full MS spectrum representing the charge state envelope of intact tRNA^{PHE} from -26 to -9. (B) Zoomed-in region of the most intense charge state (-17). C) Zoomed-in raw spectrum of -17 charge state demonstrating the baseline isotopic resolution of both major and minor species. (D) Annotated deconvoluted spectrum from tRNA^{PHE}. The annotation describes the variant as well as the 3' terminal identified. Note: Asterisk identifies species having the known 5' phosphate.

Table 4. Theoretical monoisotopic masses of intact tRNA^{PHE} having truncation on the 3' terminal

		5'p-CpCpA-aa	5'p-CpCpA	5'p-CpCp	5'p-CpC	5'p-Cp	5'p-C
tRNA^{PHE}	A67	24989.586	24939.487	24690.401	24610.456	24385.360	24305.394
	G67	25004.597	24954.498	24874.532	24625.466	24545.480	24320.405

Table 5. All major and minor species of intact tRNA^{PHE} identified by BioPharma Finder software are summarized here, including the modifications, their theoretical and experimental monoisotopic masses, mass deviation, and relative abundance. Asterisk represents species having a 5' phosphate.

Modification	Theoretical monoisotopic mass (Da)	Experimental monoisotopic mass (Da)	Mass deviation (ppm)	Relative abundance (%)
PHE A67 *CpC	24610.456	24610.532	3.3	100.00
PHE G67 *CpC	24625.466	24626.526	3.3†	25.77
PHE A67 *CpC undermethylated yW	24596.388	24596.547	4.6	22.74
PHE A67 *CpCpA	24939.487	24939.578	3.1	18.55
PHE G67 *CpCpA	24954.498	24955.592	3.8†	4.72
PHE A67 CpC	24530.469	24530.570	4.1	<3
PHE A67 Cp	24305.394	24305.404	0.4	<3

† Chemical formula manually adjusted by 1 Da to account for A0 isotopomer overlap

Conclusion

Here, we demonstrate the utility of Orbitrap high-resolution accurate-mass mass spectrometry coupled to an ion pairing chromatography platform for the intact mass analysis of tRNA.

- High quality intact tRNA^{PHE} HRAM MS data (isotopic resolution and minimal salt adducts) were collected on the Orbitrap Ascend Tribrid mass spectrometer by combining the biocompatible UHPLC system and the use of dibutylamine solvent with the DNAPac RP column.
- The high-resolution baseline isotope resolved intact tRNA^{PHE} spectra were easily processed by Xtract deconvolution algorithm implemented in the BioPharma Finder Intact Mass Analysis workflow.
- Confident monoisotopic mass confirmations with less than 5 ppm mass accuracy were achieved for all identified tRNA^{PHE} species.
- BioPharma Finder software's flexibility and ease of use enables users to create theoretical variants for identification and results in fast data processing and annotation.
- For the analysis of tRNAs the precision in the deconvolution allows for facile discrimination between known sequences with sensitivities of single base substitutions or demethylations.

Thermo Fisher SCIENTIFIC

References

1. Boccaletto, P.; MacHnicka, M.A.; Purta, E.; Pitkowski, P.; Baginski, B.; Wirecki, T.K.; De Crécy-Lagard, V.; Ross, R.; Limbach, P.A.; Kotter, A.; Helm, M.; Bujnicki, J.M. MODOMICS: A Database of RNA Modification Pathways. 2017 Update. *Nucleic Acids Res.* **2018,** *46*(D1), D303–D307.
2. Keith, G.; Dirheimer, G. Evidence for the Existence of an Expressed Minor Variant $tRNA^{Phe}$ in Yeast. *Biochem. Biophys. Res. Commun.* **1987,** *142*(1), 183–187. https://doi.org/10.1016/0006-291X(87)90468-2
3. Vanhinsbergh, C.J.; Criscuolo, A.; Sutton, J.N.; Murphy, K.; Williamson, A.J.K.; Cook, K.; Dickman, M.J. Characterization and Sequence Mapping of Large RNA and MRNA Therapeutics Using Mass Spectrometry. *Anal. Chem.* **2022,** *94*(20), 7339–7349. https://doi.org/10.1021/acs.analchem.2c00765
4. Ross, R. L.; Murphy, K.; Zheng, Y.; Du, M. Thermo Fisher Scientific Application Note 001108: Characterization of MRNA 5' Capping Products Using an LC-HRAM-MS/MS Analytical Platform and Thermo Scientific BioPharma Finder Software Solution. https://assets.thermofisher.com/TFS-Assets/CMD/Application-Notes/an-001108-pb-lc-hram-ms-mrna-quantification-an001108-na-en.pdf
5. Kebarle, P.; Tang, L. From Ions in Solution to Ions in the Gas Phase - the Mechanism of Electrospray Mass Spectrometry. *Anal. Chem.* **1993,** *65* (22), 972A–986A. https://doi.org/10.1021/ac00070a001
6. Kebarle, P.; Verkerk, U. H. Electrospray: From Ions in Solution to Ions in the Gas Phase, What We Know Now. *Mass Spectrom. Rev.* **2009,** *28* (6), 898–917. https://doi.org/10.1002/mas.20247
7. Apffel, A.; Chakel, J. A.; Fischer, S.; Lichtenwalter, K.; Hancock, W. S. Analysis of Oligonucleotides by HPLC−Electrospray Ionization Mass Spectrometry. *Anal. Chem.* **1997,** *69* (7), 1320–1325. https://doi.org/10.1021/ac960916h
8. Jarrous, N.; Reiner, R. Human RNase P: A tRNA-Processing Enzyme and Transcription Factor. *Nucleic Acids Res.* **2007,** *35*(11), 3519–3524. https://doi.org/10.1093/nar/gkm071
9. RajBhandary, U.; Chang, S.; Stuart, A.; Faulkner, R.; Hoskinson, R.; Khorana, H. Studies on Polynucleotides, Lxviii* the Primary Structure of Yeast Phenylalanine Transfer RNA. *Proc. Natl. Acad. Sci.* **1967,** *57*(3), 751–758.
10. Huang, T.-Y.; Liu, J.; McLuckey, S.A. Top-Down Tandem Mass Spectrometry of tRNA Via Ion Trap Collision-Induced Dissociation. *J. Am. Soc. Mass Spectrom.* **2010,** *21*(6), 890–898. https://doi.org/10.1016/j.jasms.2009.12.007
11. Noma, A.; Kirino, Y.; Ikeuchi, Y.; Suzuki, T. Biosynthesis of Wybutosine, a Hyper-Modified Nucleoside in Eukaryotic Phenylalanine tRNA. *EMBO J.* **2006**, *25*(10), 2142–2154. https://doi.org/10.1038/sj.emboj.7601105

General Laboratory Equipment – Not For Diagnostic Procedures. © 2023 Thermo Fisher Scientific Inc. All rights reserved. All trademarks are the property of Thermo Fisher Scientific and its subsidiaries unless otherwise specified. This information is presented as an example of the capabilities of Thermo Fisher Scientific products. It is not intended to encourage use of these products in any manner that might infringe the intellectual property rights of others. Specifications, terms and pricing are subject to change. Not all products are available in all countries. Please consult your local sales representative for details. **AN002601-EN 1223S**

thermo scientific