MobiliTraIN

## Ion Mobility Mass Spectrometry Training Network

Project no. 101119562

# Deliverable 6.1
# Temperature calibrant molecules for IM-HRMS

**Version** 1.0

WP 6 – **Fundamentals - Fundamentals and standardisation**

| | |
|---|---|
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# Executive Summary


## Background

Deliverable D6.1 is part of WP6 (Fundamentals and Standardization) and addresses Task 6.1: Establishment of Temperature Calibrants for Instrument Tuning. The overall goal is to identify suitable temperature-sensitive molecules or molecular complexes that dissociate or unfold at well-defined activation conditions, enabling a practical tuning method to translate activation/temperature conditions between instrumental platforms.

## Objectives

- Propose a practical set of commercially available calibrant molecules for both ionization polarities that cover a range of compound classes, molecular masses and energetics.

- Define how the list of the temperature calibrants is used for tuning conditions across different IM-HRMS platforms.

## Methodology and Implementation

Candidate molecules were selected from the literature using the following criteria: (i) commercial availability; (ii) a simple and interpretable activation readout (e.g., single-step fragmentation, a dominant neutral loss, complex dissociation, or a reproducible unfolding transition or compaction); and (iii) coverage across positive and negative ionization modes, compound classes, and a broad mass range from small molecules to large proteins.

## Outcomes

The calibrant panel enables a reproducible and transparent approach to establish IM-HRMS tuning conditions. It lays the foundation for harmonized reporting of ion preparation and activation across different platforms, reduces activation-driven artefacts (unintended fragmentation or structural rearrangements), and provides a common basis for interlaboratory comparisons.

## Next Steps

The selected calibrant molecules will be tested across TWIMS, TIMS, and DTIMS instruments to define optimal tuning conditions, using consistent readouts such as precursor survival yields, minimal unintended fragmentation, stable mobility features or compaction upon activation. The resulting tuning protocol will then be validated across multiple laboratories within the MobiliTraIN consortium to assess reproducibility and support inter-platform harmonization.

# Revision History

| Author(s) | Description | Date |
|---|---|---|
| Mathew Bejoy (FUB)<br>Niklas Geue (FUB)<br>Kevin Pagel (FUB) | Deliverable draft | 20 January 2026 |
| Valérie Gabelica (UNIGE) | Revision 1 | 30 January 2026 |
| Niklas Geue (FUB) | Revision 2 | 30 January 2026 |
| Tim Causon (BOKU) | Final version | 30 January 2026 |

# Contents

# Abbreviations

| Abbreviations | Details |
|---|---|
| IM-HRMS | Ion mobility-high resolution mass spectrometry |
| IMS | Ion mobility spectrometry |
| CCS | Collision cross section |
| E0 | Threshold Dissociation Energy |
| TWIMS | Travelling wave ion mobility spectrometry |
| TIMS | Trapped ion mobility spectrometry |
| DTIMS | Drift-tube ion mobility spectrometry |
| CIU | Collision-induced unfolding |
| SY | Survival yield |
| FUB | Freie Universitaet Berlin |
| UNIGE | Universite de Geneve |

# 1 Introduction

The objectives of WP6 (Fundamentals and Standardisation) are to harmonise IM-HRMS measurements across the community. In this context, D6.1 is a set of temperature calibrants for IM-HRMS instruments. The specific objective is to have a benchmark molecular dataset for setting up reproducible IM-HRMS results.

Ion temperature and internal energy acquired during ionization, transmission and mobility separation can drive unwanted fragmentation, dissociation of noncovalent complexes, or changes in gas-phase conformational ensembles. These effects complicate the interpretation of IM-HRMS measurements and can reduce inter-platform comparability of CCS data. Temperature calibrant molecules (thermometer ions) provide practical 'sensors' for activation/temperature by offering reproducible mass or ion mobility readout under distinct tuning conditions.

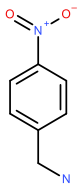
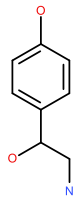
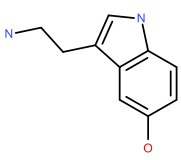
This will enable the participating groups as well as external users to facilitate studies of the impact of ion activation on CCS measurement of ten selected molecular ions assessed (MS9), but also will feed WP3 for D3.1 (Optimized IM-MS parameters for ion preparation), as the proposed temperature calibrants will be used by DC1, DC2, DC3, and DC11 in their respective projects.

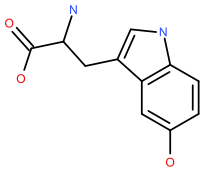
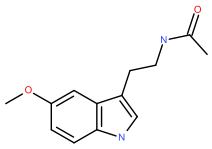
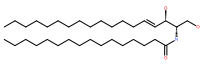
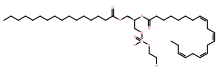
# 2 Selection Criteria and Readouts

Calibrants were prioritized when they (i) are commercially available, (ii) generate a simple, dominant readout upon activation, and (iii) represent distinct chemical classes and size scales. Readouts are defined as: fragmentation in defined dissociation channels and CCS distribution changes (compact fraction and emergence of unfolded states).

# 3 List of the Temperature Calibrants

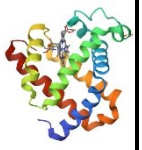
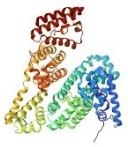
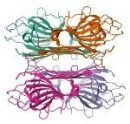
## 3.1 Positive Mode (Small Molecules and Lipids)

| | Name | Structure | Mass (Da) | $E_0$ | Readout | Reference |
|---|---|---|---|---|---|---|
| 1 | 4-Nitrobenzylamine $[M+H]^+$ |  | 152 | $E_{0\ (theor)}$ = 1.96 eV | $[M+H-NH_3]^+$ at 135 Da<br><br>Neutral loss of $NH_3$ upon activation | 10.1021/jasms.0c00151 |
| 2 | Octopamine $[M+H]^+$ |  | 153 | $E_{0\ (theor)}$ = 1.04 eV | $[M+H-H_2O]^{+*}$ at 135 Da<br><br>Neutral loss of $H_2O$ upon activation | 10.1002/jms.4802 |
| 3 | Serotonin $[M+H]^+$ |  | 176 | $E_{0\ (theor)}$ = 1.22 eV | $[M+H-NH_3]^+$ at 159 Da<br><br>Neutral loss of $NH_3$ upon activation | 10.1039/D0AN02069A |

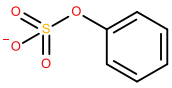
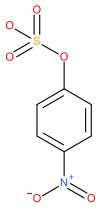
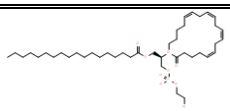
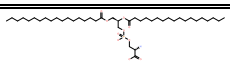
| | Name | Structure | Mass (Da) | $E_0$ | Readout | Reference |
|---|---|---|---|---|---|---|
| 4 | 5-Hydroxytryptophan $[M+H]^+$ |  | 220 | $E_{0\ (theor)}$ = 1.30 eV | $[M+H-NH_3]^+$ at 203 Da<br><br>Neutral loss of $NH_3$ upon activation | 10.1039/D0AN02069A |
| 5 | Melatonin $[M+H]^+$ |  | 232 | $E_{0\ (theor)}$ = 1.745 eV | $[M+H-C_2H_4NO]^+$ at 176 Da<br><br>Neutral loss upon activation | 10.1039/D0AN02069A |
| 6 | Ceramide $[M+H]^+$ |  | 538 | - | $[M+H-H_2O]^+$ at 520 Da<br><br>Neutral loss of $H_2O$ upon activation | 10.1021/jasms.9b00061 |
| 7 | Phosphatidylethanolamine $[M+H]^+$ |  | 740 | - | Head group loss upon activation leading to ion at 599 Da | 10.1021/jasms.9b00061 |

## 3.2 Positive Mode (Peptides and Proteins)

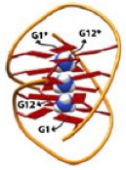
| | Name | Structure | Mass | CCS | Readout | Reference |
|---|---|---|---|---|---|---|
| 1 | Leucine enkephalin<br>z = +1 | YGGFL | 556 Da | $^{DT}CCS_{N2}$ = 228.6 Å$^2$ | Fragmentation to b4 ion | 10.1002/mas.20279 |
| 2 | Bradykinin<br>z = +3 | RPPGF SPFR | 1.06 kDa | $^{DT}CCS_{N2}$ = 447.6 Å$^2$ | Neutral loss of $H_2O$ upon activation to $[M+3H-H_2O]^{3+}$ | 10.1021/ja9609157 |
| 3 | Substance P<br>z = +3 | RPKPQQ FFGLM-NH2 | 1.34 kDa | 2 populations: $^{TIMS}CCS_{N2}$ = 440 and 500 Å$^2$ | Conformer change upon mild activation to larger conformer | 10.1021/ac501261h |
| 4 | Ubiquitin<br>z = +6<br>z = +7 |  | 8.6 kDa | Native z = +6: $^{DT}CCS_{N2}$ = 1214 Å$^2$<br><br>Native z = +7: | CCS Increase upon Activation (Unfolding of the Protein) | 10.1021/acs.analchem.0c00772 |

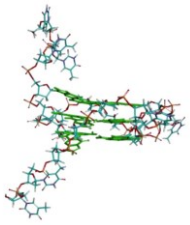
| | Name | Structure | Mass | CCS | Readout | Reference |
|---|---|---|---|---|---|---|
| | | | | $^{DT}CCS_{N2}$ = 1269 Å$^2$<br><br>Unfolded z = +7:<br>$^{DT}CCS_{N2}$ = 1851 Å$^2$ | | |
| 5 | Cytochrome C<br>z = +7 |  | 12.4 kDa | 2 populations:<br>$^{DT}CCS_{N2}$ = 1481 and 1540 Å$^2$ | CCS Increase upon Activation (Unfolding of the Protein) | 10.1021/acs.analchem.3c03788<br>10.1021/acs.analchem.7b01736 |
| 6 | Myoglobin<br>z = +8 |  | 17.6 kDa | Native:<br>$^{DT}CCS_{N2}$ = 1934 Å$^2$ | 1. Loss of Heme Group that Appears at 617 Da<br>2. CCS Increase upon Activation (Unfolding of the Protein) | 10.1021/acs.analchem.3c03788<br><br>10.1016/S1044-0305%2897%2900010-X% |
| 7 | BSA<br>z = +16 |  | 66 kDa | Native:<br>$^{DT}CCS_{N2}$ = 4445 Å$^2$ | CCS Increase upon Activation (Unfolding of the Protein) | 10.1021/acs.analchem.9b05130 |
| 8 | Concanavalin A<br>z = +20 |  | 104 kDa | Native:<br>$^{DT}CCS_{N2}$ = 5913 Å$^2$ | CCS Increase upon Activation (Unfolding of the Protein) | 10.1021/acs.analchem.9b05130 |
| 9 | ADH<br>z = +24 |  | 147 kDa | Native:<br>$^{DT}CCS_{N2}$ = 7427 Å$^2$ | CCS De-/Increase upon Activation (Compaction and Unfolding of the Protein) | 10.1021/acs.analchem.9b05130 |
| 10 | GroEL Tetradecamer<br>z = +69 |  | 801 kDa | Native:<br>$^{TW}CCS_{N2}$ = 219 nm$^2$ | CCS De-/Increase upon Activation (Compaction and Unfolding of the Protein) | 10.1021/ac1022953 |

## 3.3 Negative Mode (Small Molecules and Lipids)

| | Name | Structure | Mass (Da) | Energy | Readout | Reference |
|---|---|---|---|---|---|---|
| 1 | Phenyl Sulfate<br>[M-H]$^-$ |  | 173 | $E_{o\ (theor)}$ = 2.17 eV | [M-H-$SO_3$]$^-$<br>Neutral loss of $SO_3$ upon activation | 10.1021/jasms.2c00321 |
| 2 | 4-Nitrophenyl sulfate<br>[M-H]$^-$ |  | 219 | $E_{o\ (theor)}$ = 2.68 eV | [M-H-$SO_3$]$^-$<br>Neutral loss of $SO_3$ upon activation | 10.1021/jasms.2c00321 |
| 3 | Phosphatidylethanolamine<br>[M-H]$^-$ |  | 766 | - | [M-H-$C_2H_7NO_4P$]$^-$<br>Head group loss upon activation | 10.1039/C5AY00776C |
| 4 | Phosphatidylserine<br>[M-H]$^-$ |  | 792 | - | [M-H-$C_3H_5O_2N$]$^-$<br>Head group loss upon activation | 10.1039/C5AY00776C |

## 3.4 Negative Mode (Oligonucleotides)

| | Name | Structure | Mass (Da) | Readout | Reference |
|---|---|---|---|---|---|
| 1 | DDD (Dickerson-Drew dodecamer)<br>z = -5 |  <br>$(dCGCGAATTCGCG)_2$ | 7290 | Dissociates into Single Strands | 10.1007/978-1-60327-418-0_6 |
| 2 | G4T4G4<br>z = -5 |  <br>$[(dGGGGTTTTGGGG)_2 \bullet (NH_4^+)_3]$ | 7600 | Loss of Ammonium Ions at Low Energies | 10.1007/s13361-018-2029-4 |

| | Name | Structure | Mass (Da) | Readout | Reference |
|---|---|---|---|---|---|
| 3 | 20G<br>z = -7 |  <br>TTTGGGTGGGTGGGTG GGTT | 6270 | Loss of Ammonium Ions at High Energies | 10.1021/acs.analchem.2c03187 |

# 4 Outlook

The selected calibrant molecules will be systematically evaluated across traveling-wave ion mobility spectrometry (TWIMS), trapped ion mobility spectrometry (TIMS), and drift-tube ion mobility spectrometry (DTIMS) platforms to establish optimal and platform-specific tuning conditions. For each instrument type, activation and transmission parameters will be carefully varied while monitoring a set of consistent and quantitative performance metrics, including precursor ion survival yields, the extent of unintended or non-diagnostic fragmentation, and the stability and reproducibility of ion mobility features. Particular attention will be paid to activation-induced mobility changes, such as ion compaction or unfolding, as sensitive indicators of excessive internal energy deposition.

Based on these measurements, a harmonized tuning protocol will be developed that balances efficient ion transmission with controlled activation across the different ion mobility technologies. This protocol will then be validated through interlaboratory studies within the MobiliTraIN consortium, involving multiple instruments and operators, to assess reproducibility, robustness, and transferability. The resulting data will provide a foundation for cross-platform comparability and support the broader goal of inter-instrument and inter-laboratory harmonization of ion mobility mass spectrometry measurements.