University of Delaware Mass Spectrometry Facility

MALDI-TOF Polymer Analysis Guide

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Background

Matrix-assisted laser desorption/ionization (MALDI) is a soft ionization technique ideal for the analysis of large molecules. As a soft technique, MALDI tends to produce ions with minimal fragmentation. It also tends to produce fewer multiply charged ions than other soft ionization methods like electrospray ionization (ESI). MALDI is conducted by laser ablation and desorption of a solid prepared sample in interaction with a suitable chemical matrix. It is commonly paired with a time-of-flight (TOF) mass spectrometer (MS) as the mass detector.

MALDI-TOF MS is well suited for polymer analysis because it has a high tolerance for salts, large analytical mass range, and resolves solubility and solvent compatibility issues present for ESI and other polymer analysis techniques. It also generally has much lower chain length bias on ionization compared to ESI, so can provide more accurate estimates of characteristic parameters.

The MALD-TOF in the lab is a Bruker microFlex LRF (337 nm nitrogen laser, 60 Hz frequency). Resolution is up to 10,000 in reflectron mode. The possible operating range for this instrument is up to 300,000 *m/z* depending on the sample and sample preparation. Analysis <200 *m/z* is usually not possible due to background. Possible analysis range can be impacted by matrix cluster interference.

In linear mode ions travel in a linear flight path to a detector. Reflectron mode has significantly improved resolution to linear mode, as an ion mirror reflects ions before they reach a different detector. This extends the flight path and minimizes the spread of flight times of ions with the same *m/z*. Linear mode must be used when analytes are not stable enough to survive reflectron mode. Generally, this means smaller molecules can be analyzed in reflectron mode (~<4000-6000 *m/z* but varies depending on structure). However, some polymers, even polymers that have low average molecular weight, are innately fragile and prone to fragmentation during analysis and may need to be analyzed in linear mode for any detection or intact/more intact detection.

See the "Alternate and Complementary Analytical Techniques" section of this document for discussion of other analytical techniques used in polymer analysis and the limitations of MALDI-TOF.

MALDI-TOF Polymer Analysis

This guide assumes previous familiarity with MALDI sample preparation. For additional resources see the sample prep guidelines sheet and the flexSeries Quickstart Manual in the lab. For guidance on data analysis of polymer mass spectra after data is generated, see the "MALDI-TOF Polymer Analysis" document.

Matrices

Some examples of MALDI matrices used in polymer analysis are below. See the "Matrix Peaks" section of this document for guidance on *m/z* regions that may have matrix-related ion interference.

*Weighing of powders should be done with proper PPE and in a fume hood using a container and lid pre-weighing method. Follow all recommended safety precautions relevant to any chemicals used.

Name	Description
Dithranol	• Commonly used for a variety of synthetic polymers • Highest intensity matrix peaks below ~280 *m/z*
2,5-dihydroxybenzoic acid (DHB)	• Good for 700-5000 *m/z* • Commonly used for small peptides, lower mass ions, synthetic polymers • Lower energy matrix, sometimes successfully used for polymers prone to fragmentation, dendrimers • Matrix peaks below ~360 *m/z* and around 500 and 700 *m/z*
Anthracene	• Commonly used for synthetic polymers • Good for nonpolar, lower molecular weight polymers, hydrocarbon polymers
Trans-2-(3-(4-tert-Butylphenyl)-2-methyl-2-propenylidene)malononitrile (DCTB)	• Commonly used for a variety of synthetic polymers • Also good for organometallics • Aprotic matrix (protonation unlikely, generally radicals and cationized adducts)
α-cyano-4-hydroxycinnamic acid (CHCA/ACCA)	• Good for 2,000-20,000 *m/z* • Commonly used for peptides, proteins • Matrix peaks below ~1000 *m/z*
Sinapinic acid (SA)	• Good for >10,000 *m/z* • Commonly used for proteins • Matrix peaks below ~1000 *m/z*
Pencil lead	• Lower mass ions, small polymers, good for <1000 *m/z* • Quick and simple to test, eliminates solvent incompatibility • Matrix peaks depend on specific composition/binders • Graphite is also used as a matrix

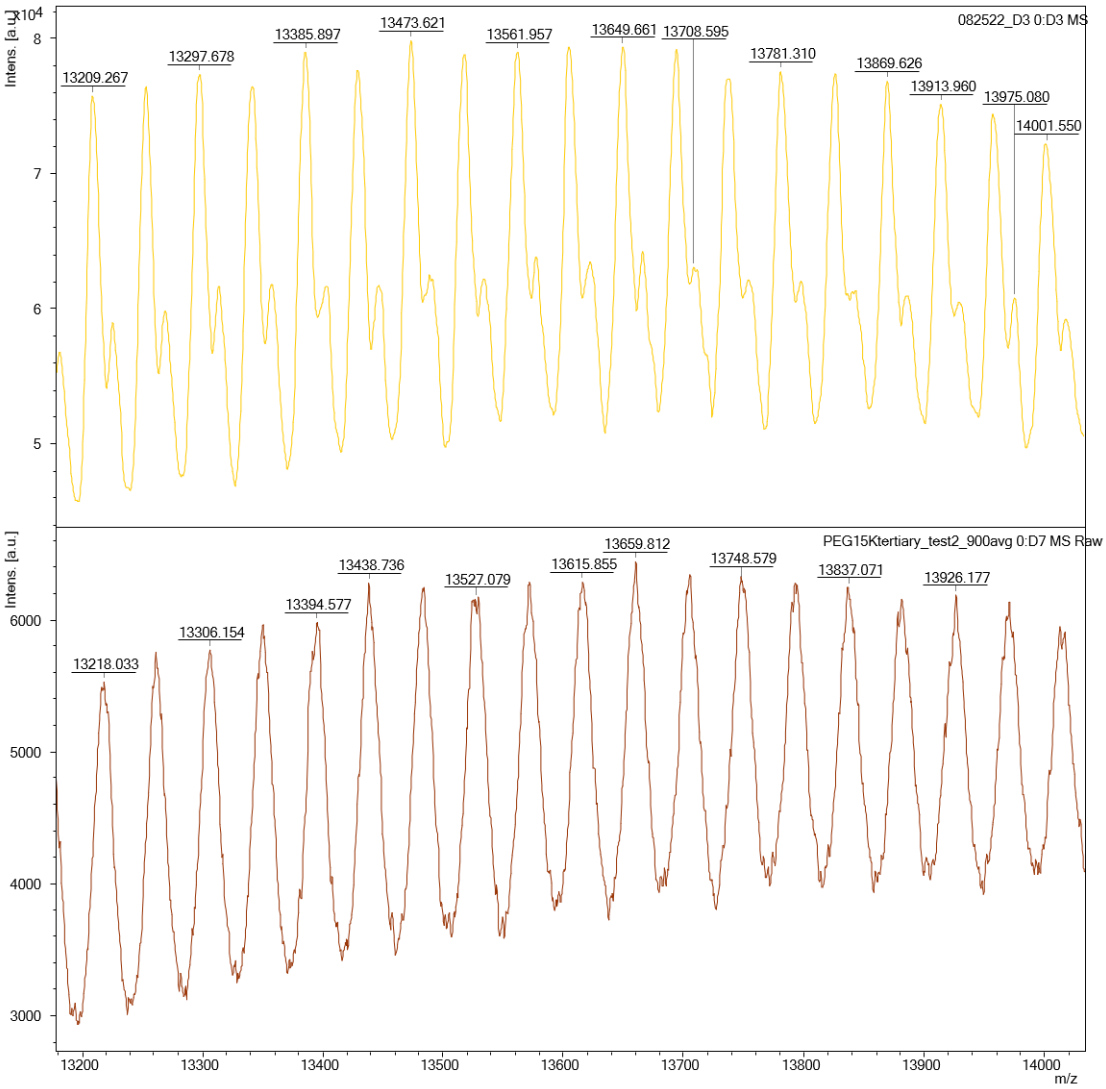
- Many other MALDI matrices are used in polymer analysis beyond this list: 2-(4-hydroxyphenylazo)benzoic acid (HABA), 3-Indoleacrylic Acid (IAA), etc., and new MALDI matrices are always being identified or developed for specific uses
- Matching polarity for matrix and polymer can be good for ionization (e.g. more polar matrix like dithranol for polar polymer like polystyrene, nonpolar matrix like anthracene for nonpolar hydrocarbon polymer like polybutadiene) - but many factors go into ionization and no universal approach
- Matrices are sometimes used in combination together, such as in binary or tertiary matrices
- A slanted baseline can indicate the need to shift the matrix:sample ratio more towards matrix
- If graphene pencil ("pencil lead") is used as a matrix, it is important to prepare matrix control spots because the clay/wax binders (often polymers) used vary by brand and graphite grade
- Consideration of matrix peaks can be more important for polymer analysis because of the larger covered *m/z* range and the need for continuous and unbiased signal across the sample range for accurate calculation of polymer characteristic parameters
- **Dithranol, DHB, or DCTB are good choices for starting test matrices for many polymers**

Cationizing Agents

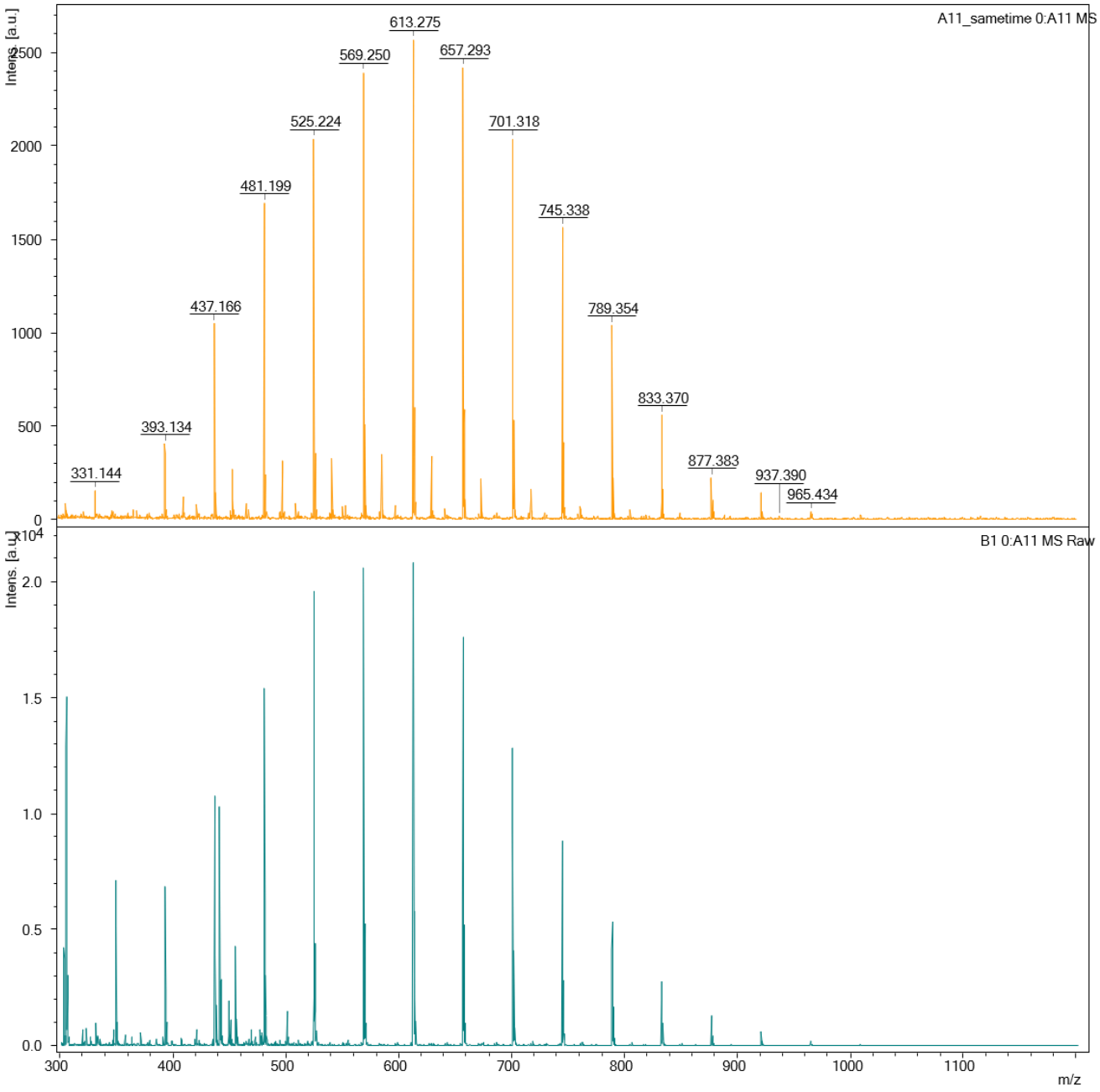
- Cationizing agents are additives to promote positive mode adduct ionization
- Many polymers more favorably ionize in other adduct forms than protonation ($[M+H]^+$) e.g. $[M+Na]^+$, $[M+K]^+$, $[M+Ag]^+$, $[M+Li]^+$
- Examples of cationizing agents include trifluoroacetic acid (TFA) (to aid protonation), NaI, CsI, KI, NaCl, LiCl, AgTFA, Ag(acac), $AgNO_3$, $CuCl_2$, $Cu(NO_3)_2$, and many others
- Adduct ionization is common even without additions to promote it. Salts like Na^+, K^+, Cl^- are common contaminants from glass and other materials used during sample storage and preparation or may already be present on the plate
- Certain polymers have typical additives applied for successful cationization such as soft metal ions like Ag and Cu for nonpolar polymers (e.g. Ag for polystyrene) and alkali metals like Na, K, Cs for polyethylene glycols and many other polymers
- Polar matrices with Ag as an additive can form silver cluster ions up to high m/z (up to 7000+ m/z) (Macha et al., 2001). Ag may also not be able to be fully cleaned from the MALDI plate, but the use of aluminum oxide grit can aid removal. See Macha et al. (2001) for more on silver persistence and removal.
- The counter ion can affect things like crystallization, ionization behavior, e.g. for sodium adduct formation there may be differences seen with addition of NaI vs. NaCl vs. NaTFA
- Additives can also be used to shift ionization to specific adduct forms—this can remove ionization competition, simplify spectrum complexity, isolation, and data interpretation

- Ionization may not be exclusively as $[M + X]^+$ where X is the cationic species, but may be more complicated and include other "salt cluster" adduct forms involving the metal salt and/or matrix
- The vast majority of polymers are analyzed in positive mode, though for those that preferentially ionize in negative mode, ionizing agents to form negative adducts (e.g. $[M + Cl]^-$ ions) may be used instead ("anionizing agents", though rarely used as a term)
- **A molar ratio of 10:1:1, matrix:sample:cation may be a good starting point for analysis, but can vary and requires optimization**

Some examples of polymer adduct ionization are provided below:



Zoom of PEG15k standard (M_n~13.2 kDa) with a tertiary matrix (DHB, CHCA, and SA) and no cationizing agent addition (top), showing ionization as $[M+Na]^+$ and $[M+K]^+$, and with NaTFA addition (bottom) to shift ionization to $[M+Na]^+$, minimizing spectral complexity.



PEG700 standard (M_n~645 Da) with a dithranol matrix and no cationizing agent addition (top), showing ionization as $[M+Na]^+$ and $[M+K]^+$, and with NaI addition (bottom), shifting ionization to $[M+Na]^+$. Additional background seen in this spectrum may be from the NaI used or some other contamination source.

Sample Preparation

Dried droplet	• Traditional prep method • Matrix and analyte solution are pre-mixed before being spotted onto the plate • May not be a good option if there are solvent compatibility issues between the matrix solution and analyte
Thin layer	• Matrix solution spotted onto plate and dried • Analyte solution spotted on top and dried • May help to avoid solvent incompatibility issues
Sandwich	• Same as above, but matrix spotted and dried, analyte spotted and dried, then matrix again • May help to avoid solvent incompatibility issues
Mix on plate	• Matrix solution spotted on plate • Analyte solution spotted on top before the matrix dries (or after to recrystallize) • Order of matrix and analyte addition can be reversed • Both mixed together with pipette tip to stir • Quick and helps to avoid solvent incompatibility issues • Can aid proper crystallization of analyte with matrix
Solvent-free prep	• Common for polymer analysis for the analysis of insoluble analytes • Avoids all solubility and solvent compatibility concerns

- There are many different ways to prep samples on the plate, with endless variations and naming conventions for different alterations
- Common alterations are switches in droplet order, droplet number, mixing (e.g. pre-mixed, mixed on plate), drying (e.g. on bench, forced air such as compressed air, vacuum, overnight in fume hood)

- Proper crystallization of matrix, sample, and cationizing agent (if used) is key to ionization
- Stirring the spot (e.g. with a pipet tip) can promote more homogeneous crystallization and smaller crystals, which can improve ionization signal
- The Marangoni effect and other evaporation effects can show "coffee-stain" or "bullseye" crystallization bias, where spectra from spot edges show different mass segregation or are otherwise different than spectra from the spot center – depends on analyte, solvent choices, deposition technique
- In solvent-free prep, matrix and sample are kept as solids
    - Approaches like bead-beating, mortar and pestle, and ball milling then thin deposition to the MALDI plate spot with a spatula or sometimes with the use of carbon adhesive tape to aid spotting, fall under solvent-free techniques
    - The optimal matrix:sample ratio sometimes significantly varies for solvent-free prep, with recommendations sometimes closer to 100:1 or even more highly shifted to the matrix – requires testing
- If possible, spot samples in replicates. Heterogeneous crystallization, orientation, and other factors, may make one spotting successfully ionize even when others do not
- For smaller polymers (~<1,000 *m/z*) it's worthwhile to also test pencil lead at the same time as other matrices because it is very quick to prepare – just gently scribble on spot with a dedicated MALDI pencil, blot with Kimwipe
    - if used, spot can be removed with eraser of the dedicated pencil before following typical cleaning protocol
    - requires pencil lead blank
- Sample desalting and clean-up can be done by C18 ZipTip or other SPE equivalent if needed or to control salts present to aid intentional cationization
- **NIST MALDI Recipe guide (https://maldi.nist.gov/) and previous research are sources for example MALDI recipes that are good starting points for analysis**
- **MALDI can be a bit of an art-no universal approach to analysis, particularly for polymers which are molecularly diverse and can contain many different functional groups.**
- **It's worth trying things! e.g. range of matrices, additives, different prep methods, matrix:sample ratios**
- **Polymer analysis can often involve deviations from the dried droplet method because of solubility issues or solvent incompatibility**
- **If there is little previous information available for analysis, a good starting point for many polymers is using a dried-droplet method (if the matrix and sample are in the same solvent or compatible and miscible solvents) or a layer method (if in different or incompatible solvents)**
    - **A layer method may be necessary for proper crystallization when using multiple solvents**

Calibration

- Calibration guidance is given in the flexAnalysis Manual (flexAnalysis 3.4 User Manual.pdf on the computer)
    - The cubic enhanced calibration algorithm should generally be used if minimum point requirements are met (n≥6)
- Note that any time a method is loaded, it needs to be recalibrated
- If possible, it is good practice to prepare the calibrant with the same matrix and solvent as samples; however, in polymer analysis this is often not possible due to the need for different solvents for sample preparation and the use of different typical matrices
- Internal calibration is generally not recommended because of the inherent spectral complexity in polymer analysis and the need to see a larger *m/z* range without interference
- If needed, additional internal re-calibration can be done after external calibration with *m/z* ions of exact known identity using linear correction (or higher order models if high enough n) for higher mass accuracy – see manual and Bruker guidance or ask MS facility staff
- Calibrants can be used together with a customized mass control list from both, ideally prepped together or by creating a spectra sum from multiple spots
- A custom calibration can be created with a well-characterized polymer standard if the typical peptide mix, protein standard I, and protein standard II calibrants can't be used to cover the needed mass range
- For calibration between ~390-1000 *m/z*, a custom calibration has been set up using a PEG400 and PEG700 mix (though these standards are degradable) – ask the MS facility if you are interested in using this
- Custom calibration guidance is in the manual or reach out to the MS Facility for assistance with setting up a custom calibration or editing mass control lists **(do not edit or save over existing lists)**

Sample Analysis

- Heterogeneous crystallization is very common – search the spot surface for the potential of a sample "hot-spot"
- Re-hitting the exact previously analyzed area of the spotted sample can produce a spectrum of a degraded or fragmented sample
- Bigger signal does not always equal better data! A higher signal with loss of resolution is a sign that the laser power is too high. Too high laser power can lead to artificial peak broadness, as molecules begin to be slightly off from each other when they hit the detector. This broadening will first affect larger molecules.
- If too high laser power is used during calibration this can lead to mass accuracy decreasing at higher *m/z* range.

- Spotting controls is important to be certain of sample data – matrix and matrix+cationizing agent blanks, solvent after contact with materials (pipet tips, vials, lids) with matrix, etc. This can be especially important during polymer analysis when working with solvents that can dissolve or leach plastics, as un-related polymers/plasticizers in your sample can complicate or mislead data interpretation.
- Pay attention to "materials of construction". Sometimes plastic (plastic microcentrifuge tubes, autosampler vials, micropipette tips) is needed for MALDI prep to avoid sorption concerns or salt leaching like Na, K from glassware that can affect adduct ionization. While the inverse can also be true, where glassware (e.g. glass autosampler vials, pasteur pipets) is desired or fully necessary depending on solvents used.
- Not all lab plastic is the same! While polypropylene (PP) tends to be the most common, there are multiple types of plastic that micropipette tips, tubes, etc. can come in, and tip filters are often polyethylene (PE). Make sure any plastic materials contacted are compatible and be aware of resistance ratings of different plastics to different solvents.

Troubleshooting

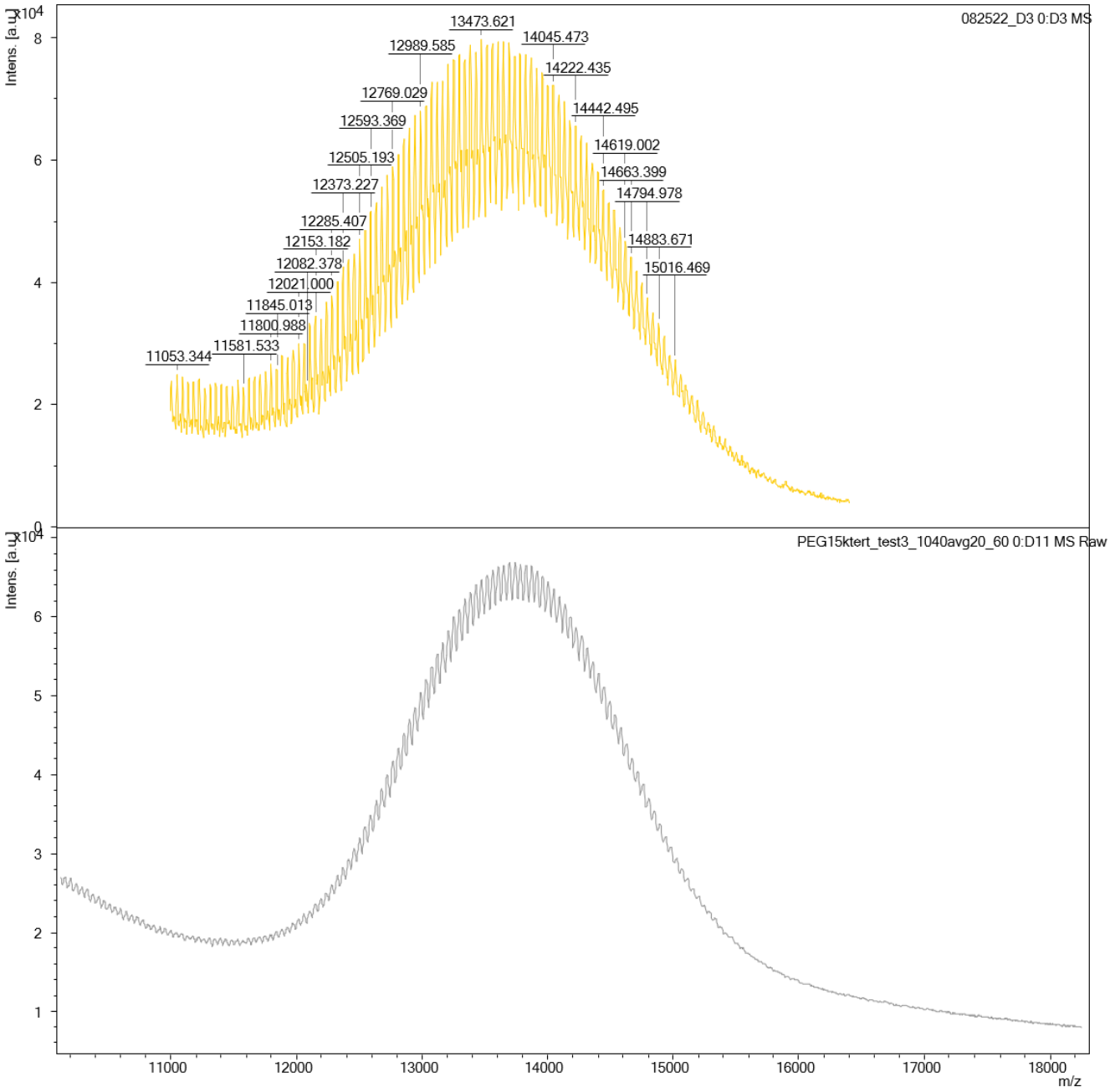
"There are a number of pitfalls during the MALDI MS analysis of a polymer sample. It is often easy to collect some mass spectral signals, but is not trivial to generate a correct mass spectrum that truly represents the chemical composition of the sample."

– Li, L. (2009). Overview of MS and MALDI MS for polymer analysis. In Li, L. (Ed.), *MALDI mass spectrometry for synthetic polymer analysis, 1-8*. John Wiley & Sons.

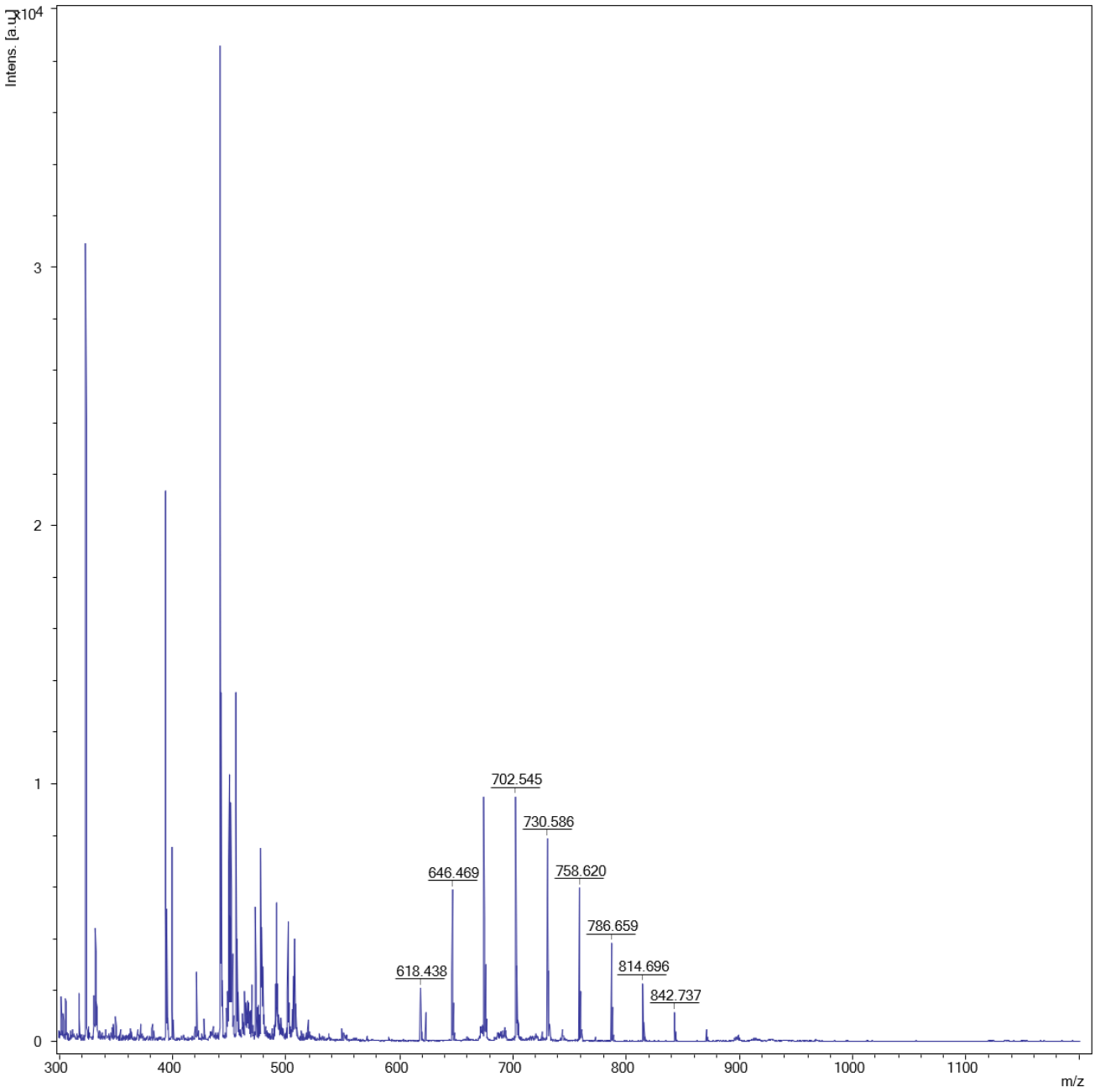
- There are many factors that can lead to a spectrum not accurately representing a polymer
    - ionization strength can show size/chain length bias or detector saturation effects
    - endgroup loss or other unintended fragmentation or alternation during the desorption/ionization process
    - mass biases related to sample drying effects, behavior in relation to the matrix, or place on the target plate
- **It is important to make sure that any generated spectrum likely accurately represents the sample or that spectral interpretation accounts for potential biases Accurate polymer average molecular weight depends on minimizing size ionization bias.**
- In some cases, it may be impossible to identify or fully account for these factors, but as possible, **it is good protocol to try multiple sample spotting protocols, solvents, and matrices, as well as to prepare spots in replicates**
- **Samples with high polydispersity (PDI ~>1.1-1.2) usually require prior molecular weight fraction separation for proper characterization of the higher mass distribution**
- **Polymer average molecular weight measured by MALDI should be considered a measured estimate and not an absolute parameter.** Additional resources that discuss these potential bias factors and MALDI polymer analysis in more detail are listed in the

references below. Different average molecular weight and distribution results can be found for different MALDI-TOF prep techniques, MALDI-TOF vs GPC, etc.

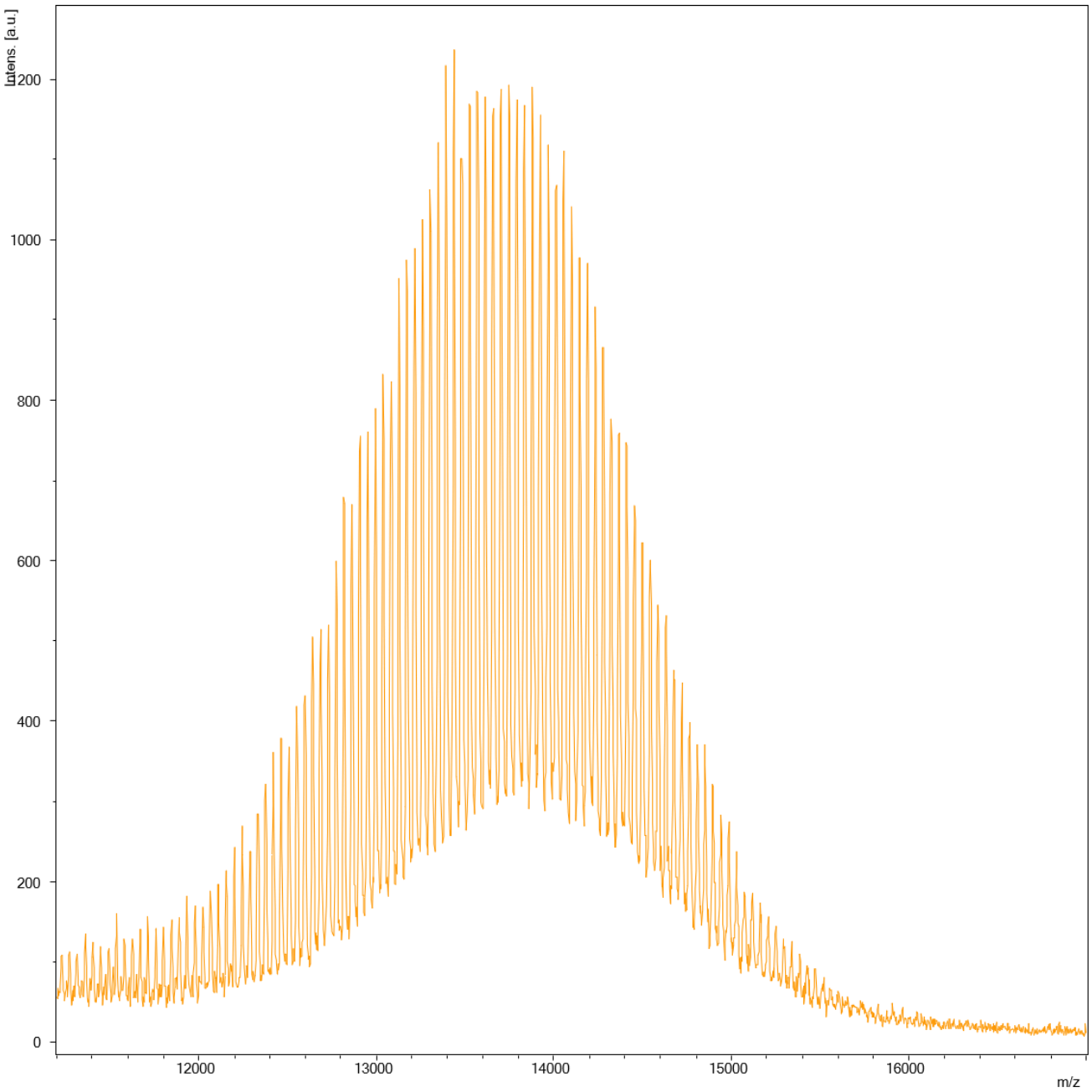
- Larger polymers may need more energy (through the use of higher energy matrices, higher laser power) to ionize, but it's a trade-off – higher energy can promote fragmentation
- Some molecules are inherently fragile and fragment even with a lower energy matrix and low laser power
- Laser power should be tested, starting low, only using the energy needed for the best spectral resolving – too high laser power = unintended fragmentation, baseline effects, peak broadening
- While most polymers ionize in positive mode, checking both ionization modes may be useful for polymers with negative ionizing functional groups. Beyond this, having data from both positive and negative mode may also help highlight background ions. For instance, positive mode may show the polymer of interest, but with overlapping *m/z* ions that do not fit the repeating unit. Sometimes those background ions are also ionizable in negative mode, confirming their difference from the polymer which is more likely to only ionize in positive mode.



PEG15k standard spectra showing the effects of too high laser energy (top) and even higher laser energy (bottom) and the corresponding loss in resolution. Laser power should be slowly ramped higher as needed, prioritizing resolution, intact detection, and spectrum/baseline shape and not signal intensity at the expense of a quality and representative spectrum.

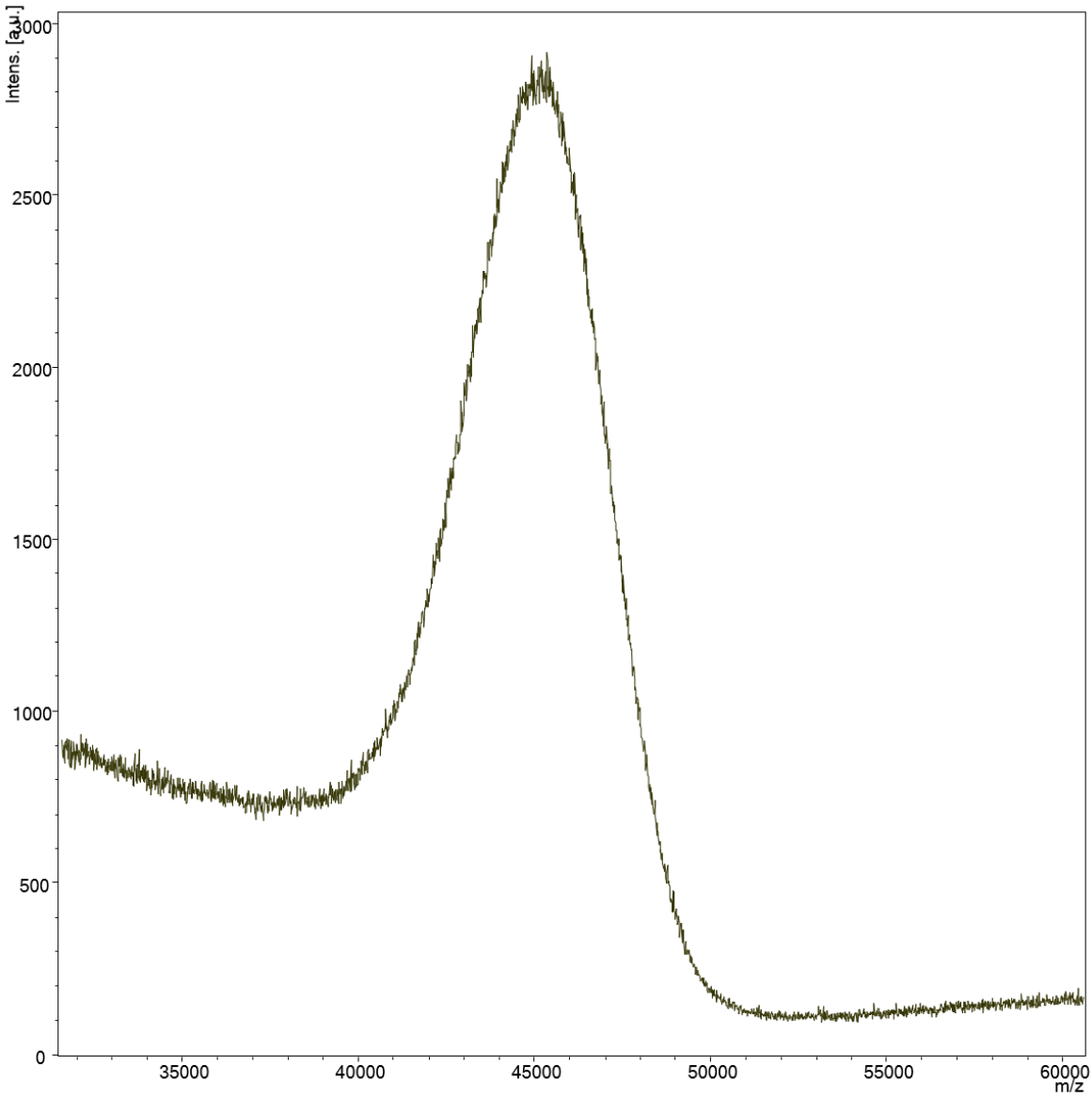


Polymer signal that was traced using control sample tests to unintended contamination from lab materials. The 28 *m/z* repeating unit appears to be a polyethylene (CH_2—CH_2) spacing.



The elevation of the PEG15k spectrum here likely partially reflects the use of higher laser power than needed, where peaks may have been better resolved at lower laser power and signal intensity. Though with lower resolving power at increasing *m/z*, increasing spectral complexity, and/or increase in signal, at some point it is expected for peaks to become unresolved and may reflect the best generatable data.

It can be important to check *m/z* ranges beyond just the expected region to understand the analysis. In this spectrum of a PEG15k standard, while the resolution of the spectrum reflects too high laser power, even at lower energy, a wide distribution of the PEG repeating unit ($O—CH_2—CH_2$) was seen. This significant lower *m/z* tail likely reflects fragmentation such as “pyrolysis” fragmentation of the standard during desorption, a known phenomenon for higher mass PEGs (Marie et al., 2000), though other factors like degradation of the standard and original standard distribution could also be reflected here.



A spectrum of a PEG50k standard (Mn~48.6 kDa) with no repeating unit resolution. The height of the spectrum to the left likely reflects fragmentation similar to seen above.

Sample Processing

Sample processing guidance is in the separate MALDI Data Analysis guidance document. While not recommended unless absolutely necessary for specific features, if you need to process outside of FlexAnalysis, external tools like MALDIquant (https://doi.org/10.1093/bioinformatics/bts447) can be used to process Bruker flex series spectra in R or other freeware options may be available. MS vendor files are generally proprietary and convertible in only one direction (vendor to open-source, not open-source to vendor).

Alternate and Complementary Analytical Techniques

Complementary Analytical Techniques

- Gel permeation chromatography (GPC) can provide average molecular weight and PDI estimates, though it has lower accuracy for lower molecular weight samples and may give inaccurate results depending on structure and representativeness of calibration standards. It is also subject to solvent and column compatibility restrictions.
- Nuclear magnetic resonance (NMR) can provide limited structural characterization, purity info, and in some cases average molecular weight estimates, but is limited in what functional groups can be analyzed and the level of structural info provided
- Spectroscopic techniques like infrared spectroscopy (IR) can also be used for polymer endgroup characterization

Separation Before Analysis

- Separation of a polymer sample before MALDI can make spectral interpretation easier or be necessary for the analysis of mass ranges that would not have been able to be detected in the original mer-distribution due to effects like detector saturation or ionization or crystallization bias issues for polydisperse samples
- Some separation examples being techniques such as liquid chromatography (LC), size-exclusion chromatography (SEC) like GPC or other SEC, other chromatography, molecular weight cutoff filtration, ion mobility spectrometry (IMS) etc.

Alternate (or Complementary) Techniques

- MALDI-MS/MS (usually TOF/TOF) is MS/MS fragmentation, so is suited for detailed analysis of branched polymers, analysis of polymers prone to fragmentation under MALDI-TOF conditions, and getting better structural information, especially for complex or larger polymers
- MALDI can be used as an ion source for Fourier transform MS (MALDI-FTMS), either Fourier transform ion cyclotron resonance (FTICR) or Orbitrap. FTMS mass analyzers are higher resolution than TOF and can allow for more detailed structural determination and better analysis of complex or fragmenting polymers. FTICR and Orbitrap detectors have trapping restrictions (surviving trapping, avoiding overfilling, etc.) and limited *m/z* detection range (~<2000 FTICR; <4000 *m/z* Orbitrap, in reality more limited, and different resolution decay with *m/z*) compared to the theoretically limitless detection range of a TOF.
- For certain analytes and research goals, other ion sources like electrospray ionization (ESI), atmospheric pressure photoionization (APPI), ambient ionization like direct analysis in real time (DART) or desorption electrospray ionization (DESI), etc. coupled to MS can be used for polymer analysis, but this is usually for lower molecular weight polymers (e.g. if not coupled to a TOF, within non-TOF detector range; solubility

requirements for ion source as relevant, etc.) and with a control for, or acceptance of, associated ionization biases (e.g. greater chain length bias, multi-charging).

- Secondary-ion MS (SIMS) like TOF-SIMS is also used in polymer analysis on polymer surfaces or dried samples on substrate

Further Reading/ References

MALDI-TOF Analysis for Polymers:

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Raeder, H. J. S. W., & Schrepp, W. (1998). MALDI-TOF mass spectrometry in the analysis of synthetic polymers. *Acta Polymerica*, *49*(6), 272-293. https://doi.org/10.1002/(SICI)1521-4044(199806)49:6<272::AID-APOL272>3.0.CO;2-1

General Prep and Analysis:

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Recognizing Alternate Ionization/Fragmentation:

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Marie, A., Fournier, F., & Tabet, J. C. (2000). Characterization of synthetic polymers by MALDI-TOF/MS: Investigation into new methods of sample target preparation and consequence on mass spectrum finger print. *Analytical chemistry*, *72*(20), 5106-5114. https://doi.org/10.1021/ac000124u

Trimpin, S. (2009). Solvent-free MALDI sample preparation. In Li, L. (Ed.), *MALDI mass spectrometry for synthetic polymer analysis, 159-186*. John Wiley & Sons.

Trimpin, S., Rouhanipour, A., Az, R., Räder, H. J., & Müllen, K. (2001). New aspects in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry: a universal solvent-free sample preparation. *Rapid Communications in Mass Spectrometry*, *15*(15), 1364-1373. https://doi.org/10.1002/rcm.372

Yoo, H. J., Kim, D. H., Shin, D., Oh, Y., Lee, S., Lee, J. Y., ... & Cho, K. (2020). Recent developments in pre-treatment and analytical techniques for synthetic polymers by MALDI-TOF mass spectrometry. *Analytical Methods*, *12*(48), 5767-5800. https://doi.org/10.1039/D0AY01729A

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Langley, G. J., Herniman, J. M., & Townell, M. S. (2007). 2B or not 2B, that is the question: further investigations into the use of pencil as a matrix for matrix-assisted laser desorption/ionisation. *Rapid Communications in Mass Spectrometry: An International Journal Devoted to the Rapid Dissemination of Up-to-the-Minute Research in Mass Spectrometry*, *21*(2), 180-190. https://doi.org/10.1002/rcm.2827

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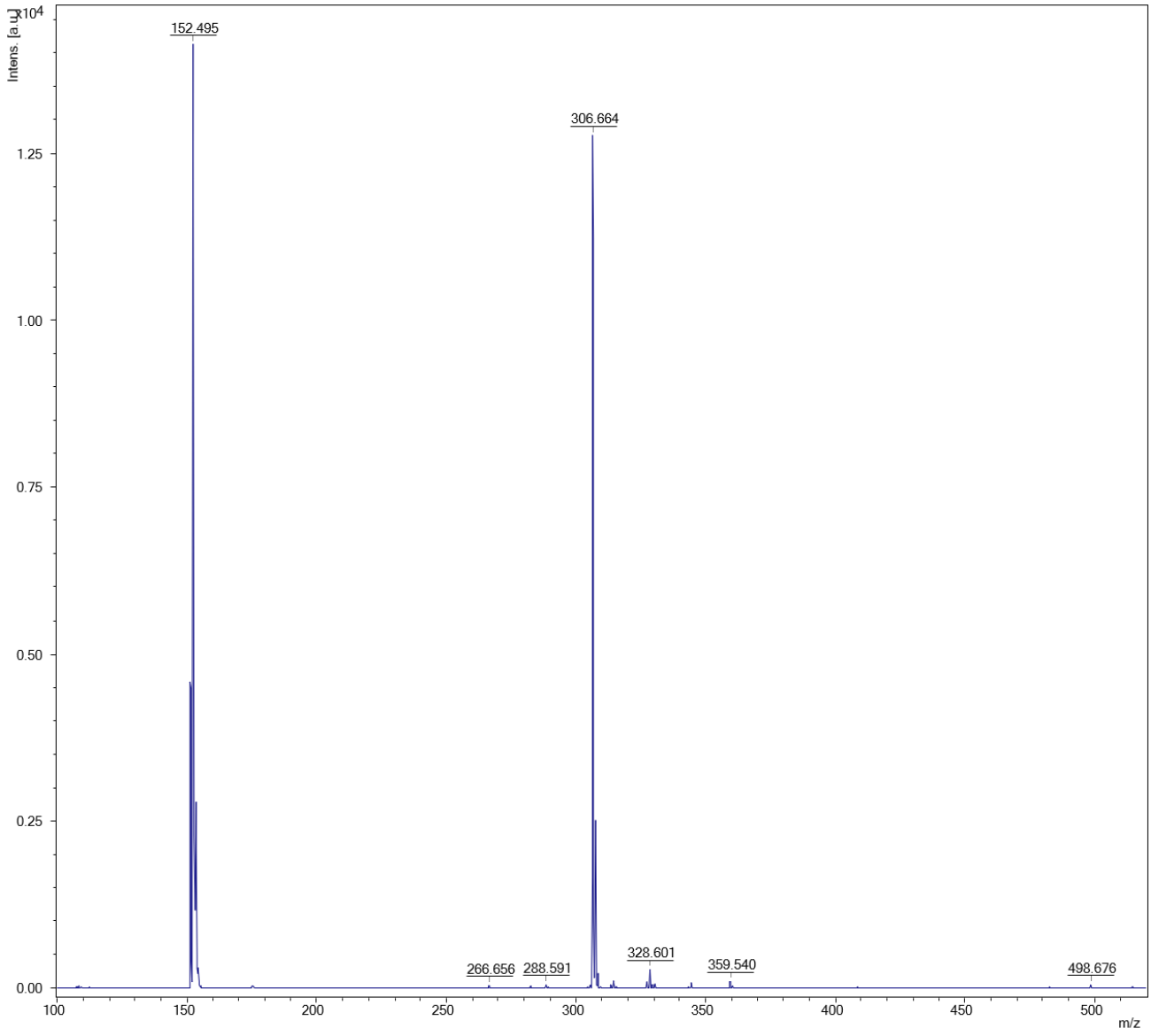
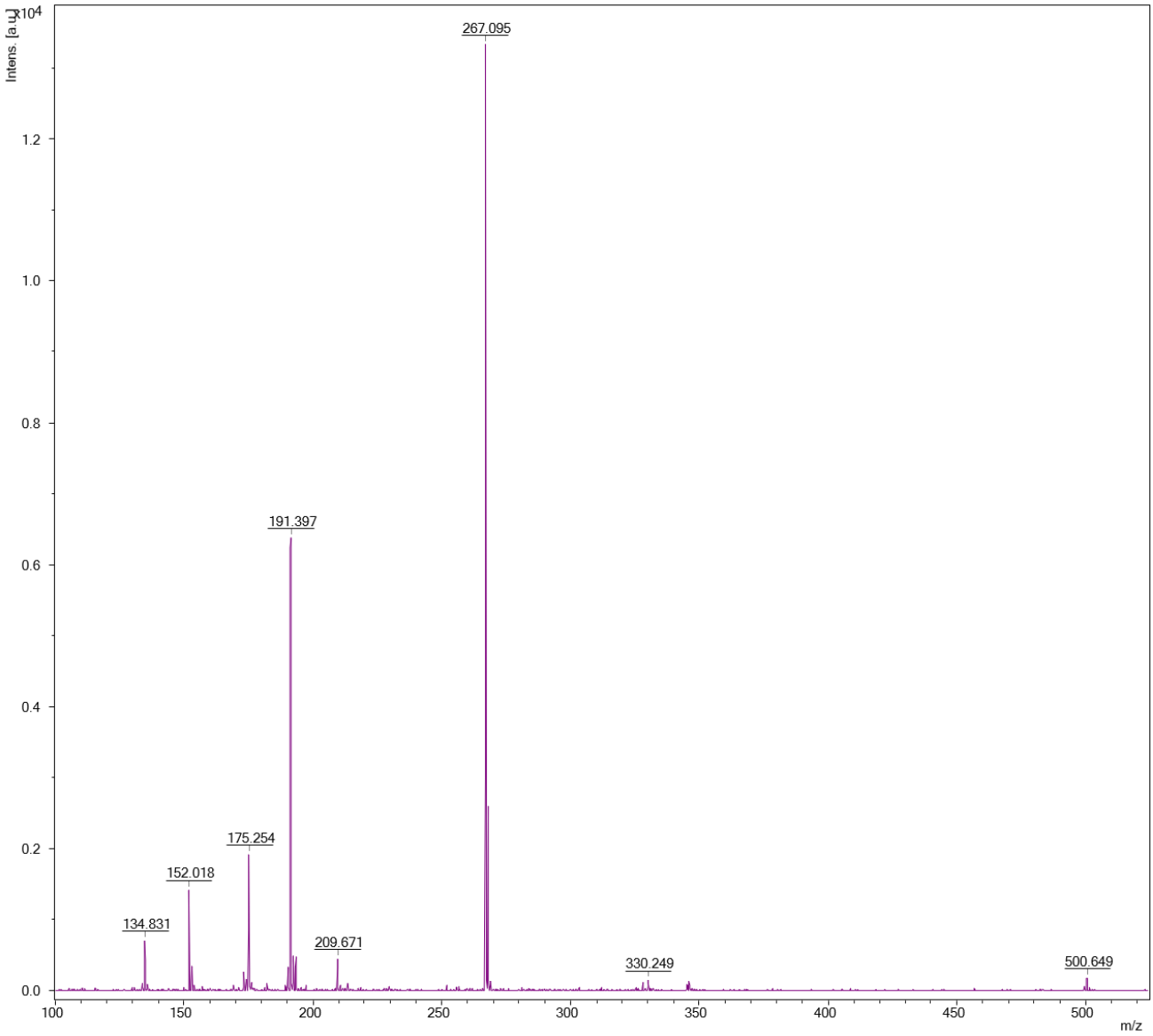
Matrix Peaks

Note: These are only example spectra generated using matrices in the lab to help guide matrix peak understanding and identification. Methods were not calibrated at time of analysis and display mass error ~±0.5-0.8 *m/z*.

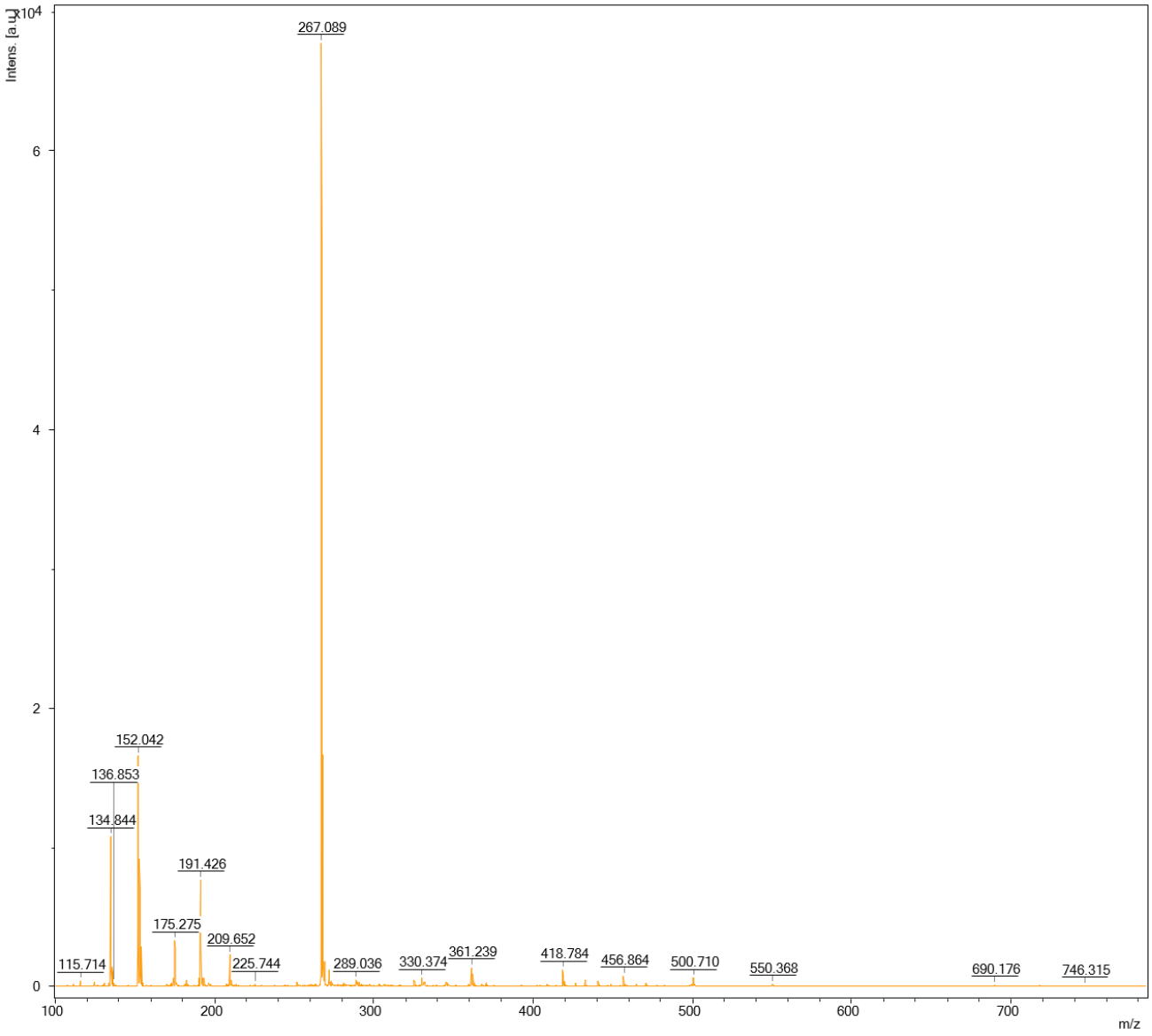
Matrix peaks vary depending on the standard, solvents, and analysis conditions used and can often form in interaction with the sample and/or cationizing agent. Matrix control spectra should be collected at time of analysis under the experimental conditions reflecting the analyzed sample(s). Matrix peaks may also be suppressed during analysis with a sample and may not be an issue even over expected *m/z* ranges with interference.



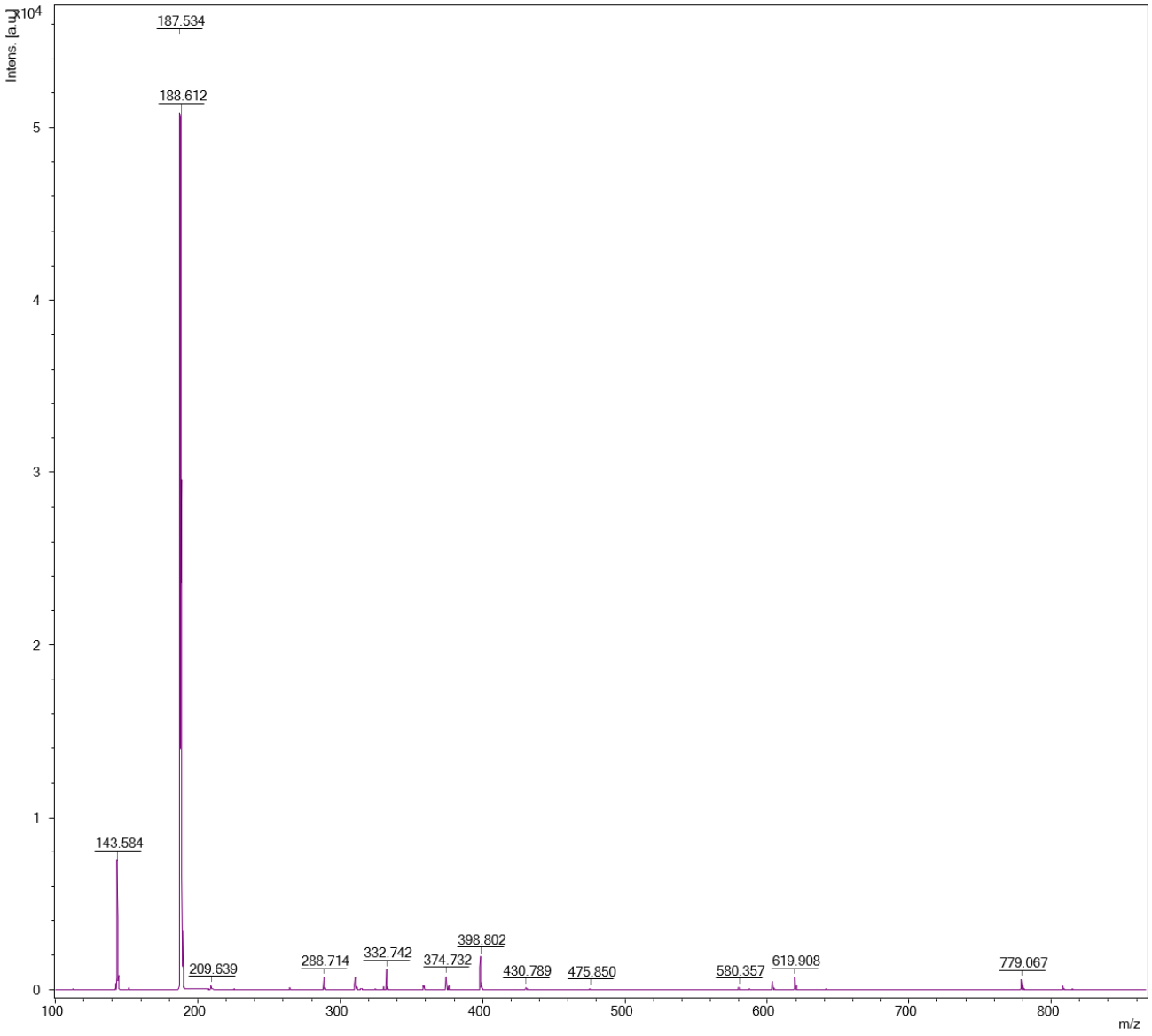
DHB matrix in reflectron negative mode



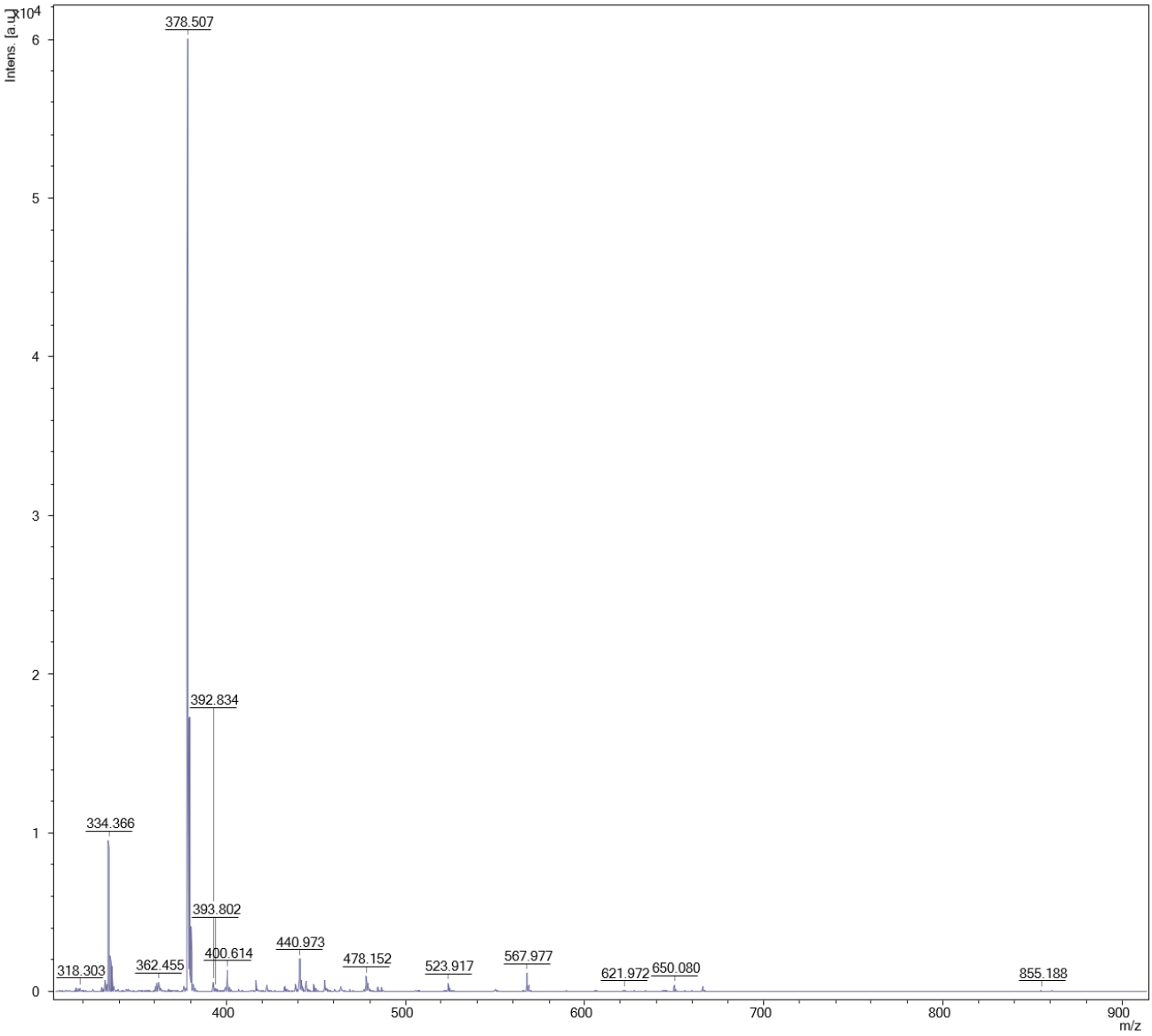
DHB matrix in reflectron positive mode



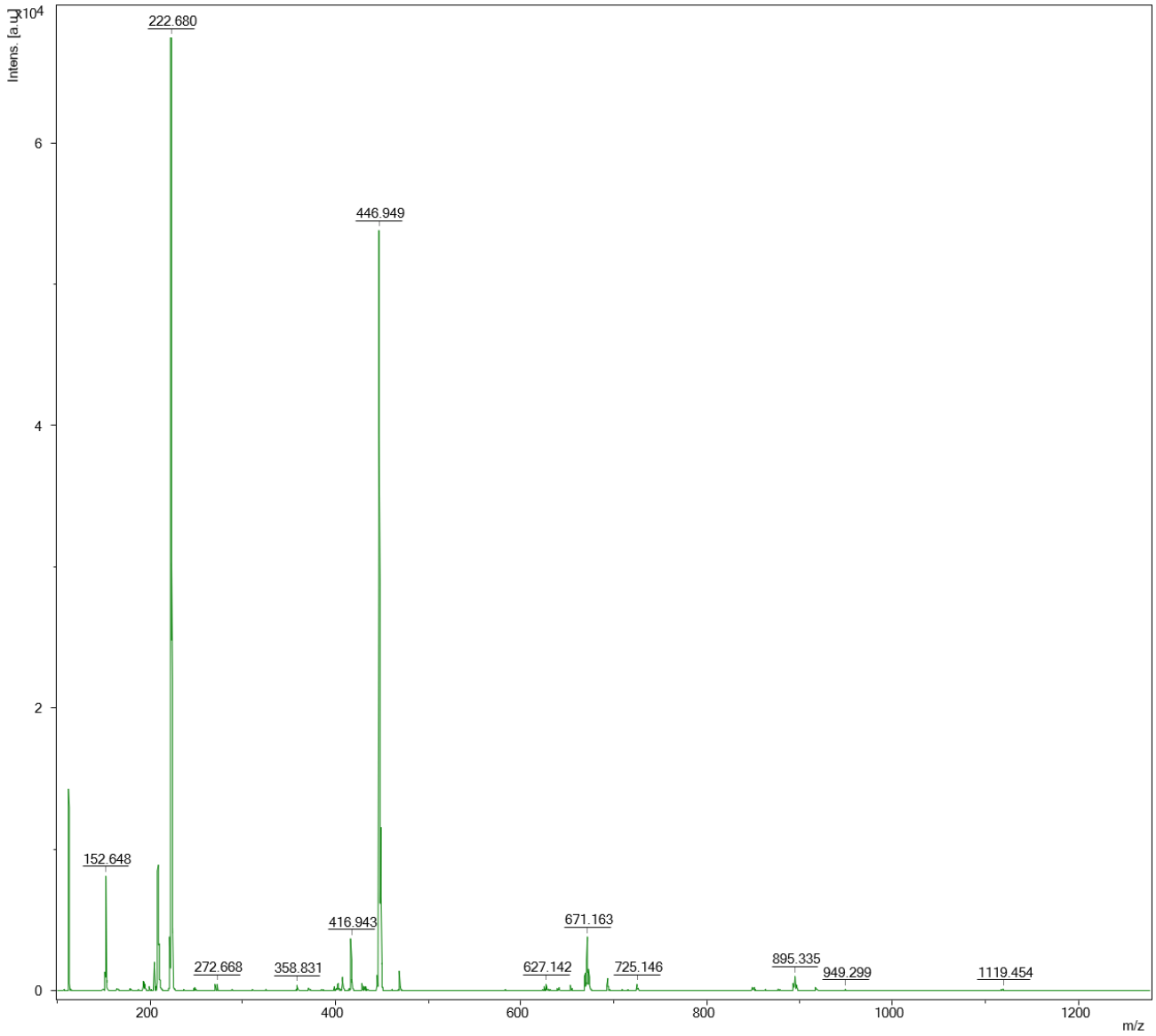
DHB matrix in reflectron positive mode at high laser power



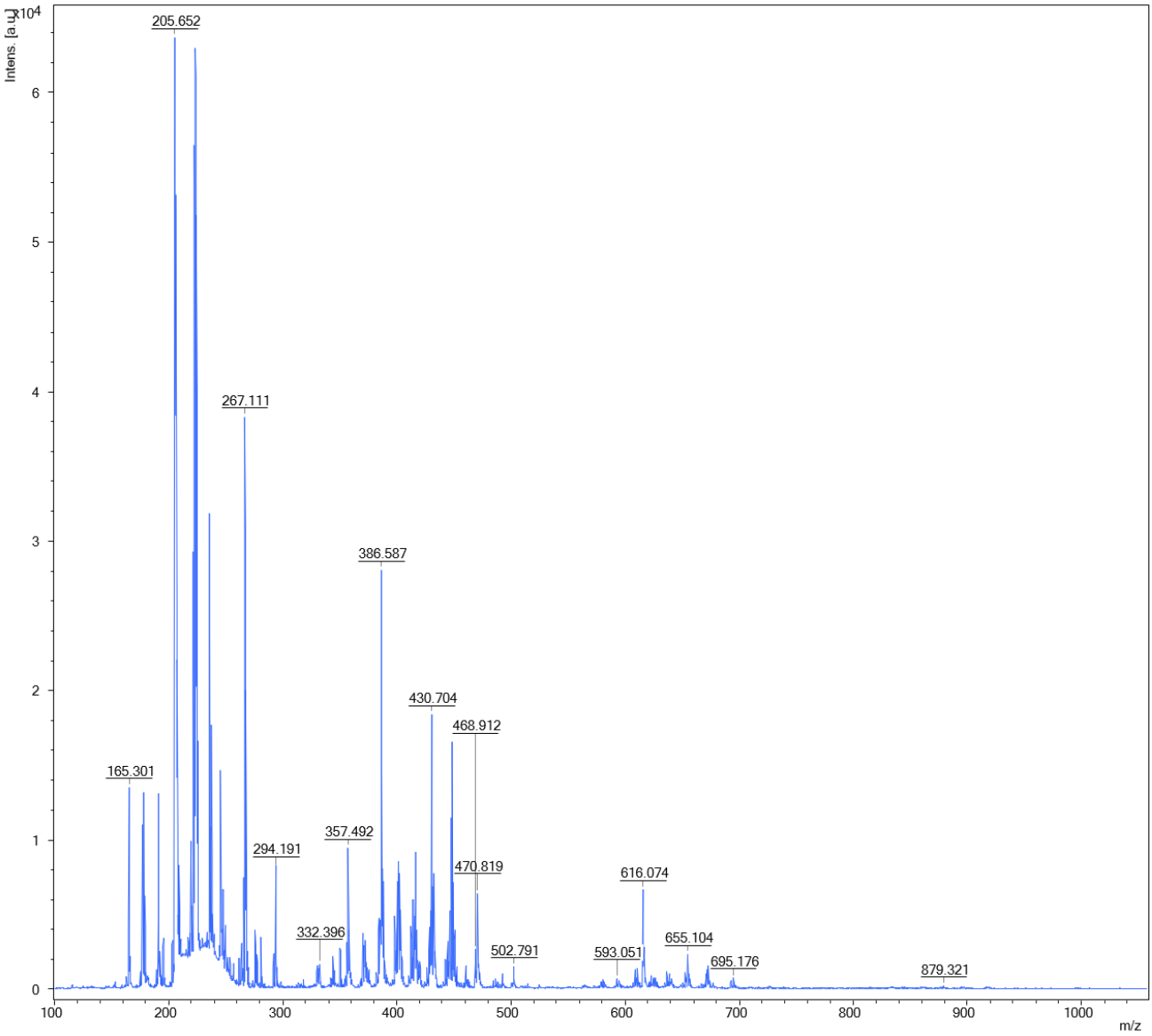
CHCA matrix in reflectron negative mode



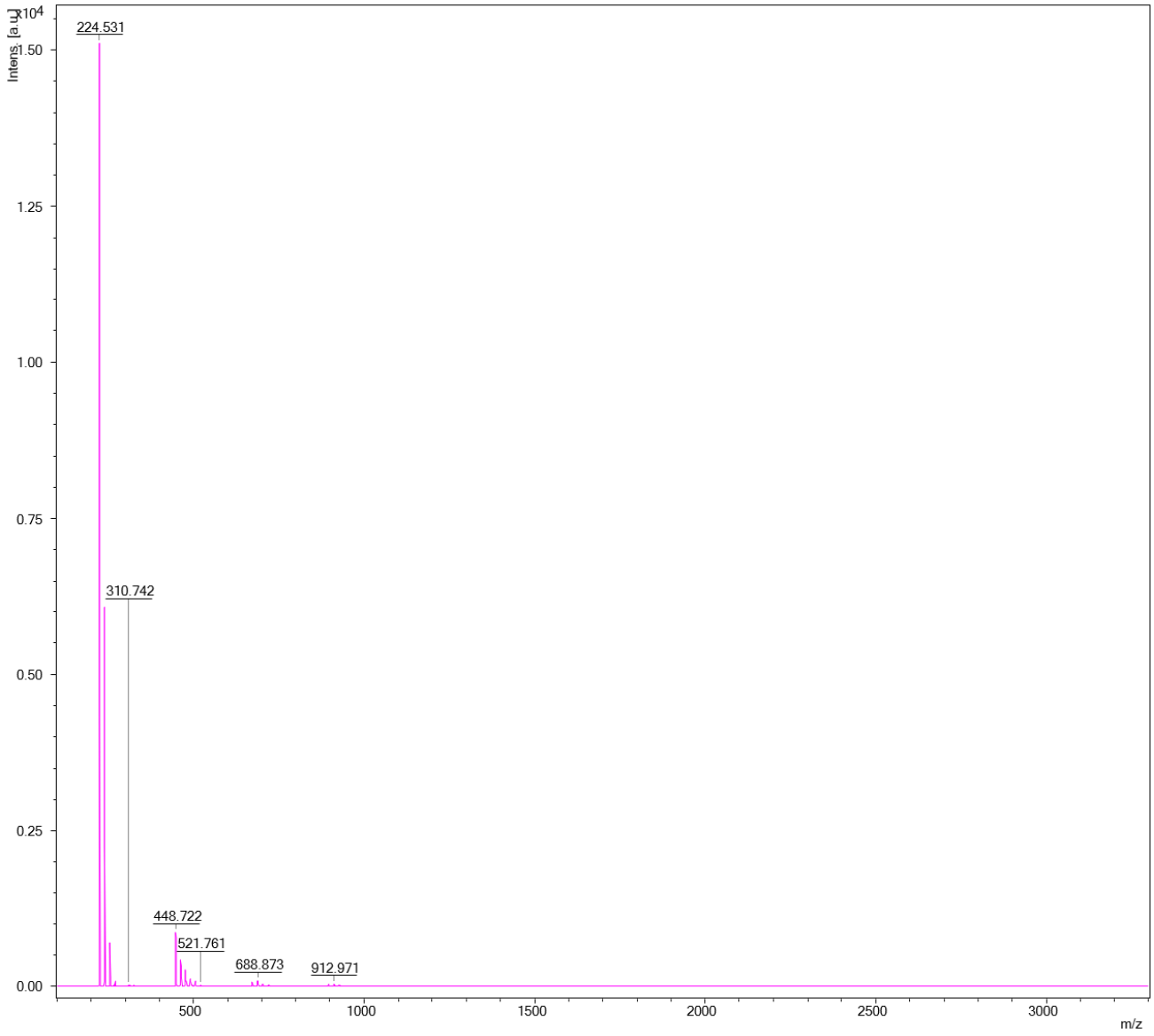
CHCA matrix in reflectron positive mode



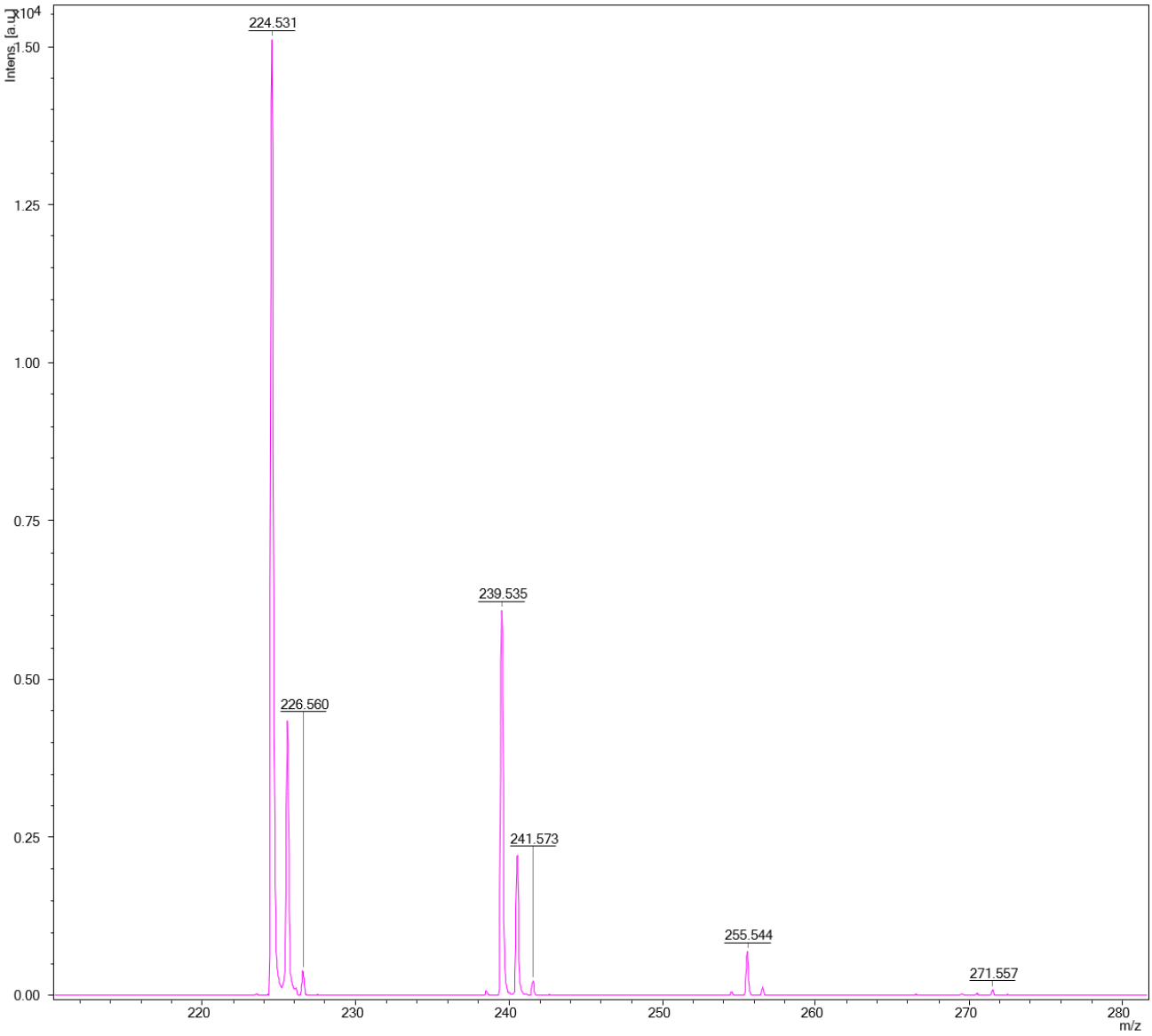
SA matrix in reflectron negative mode



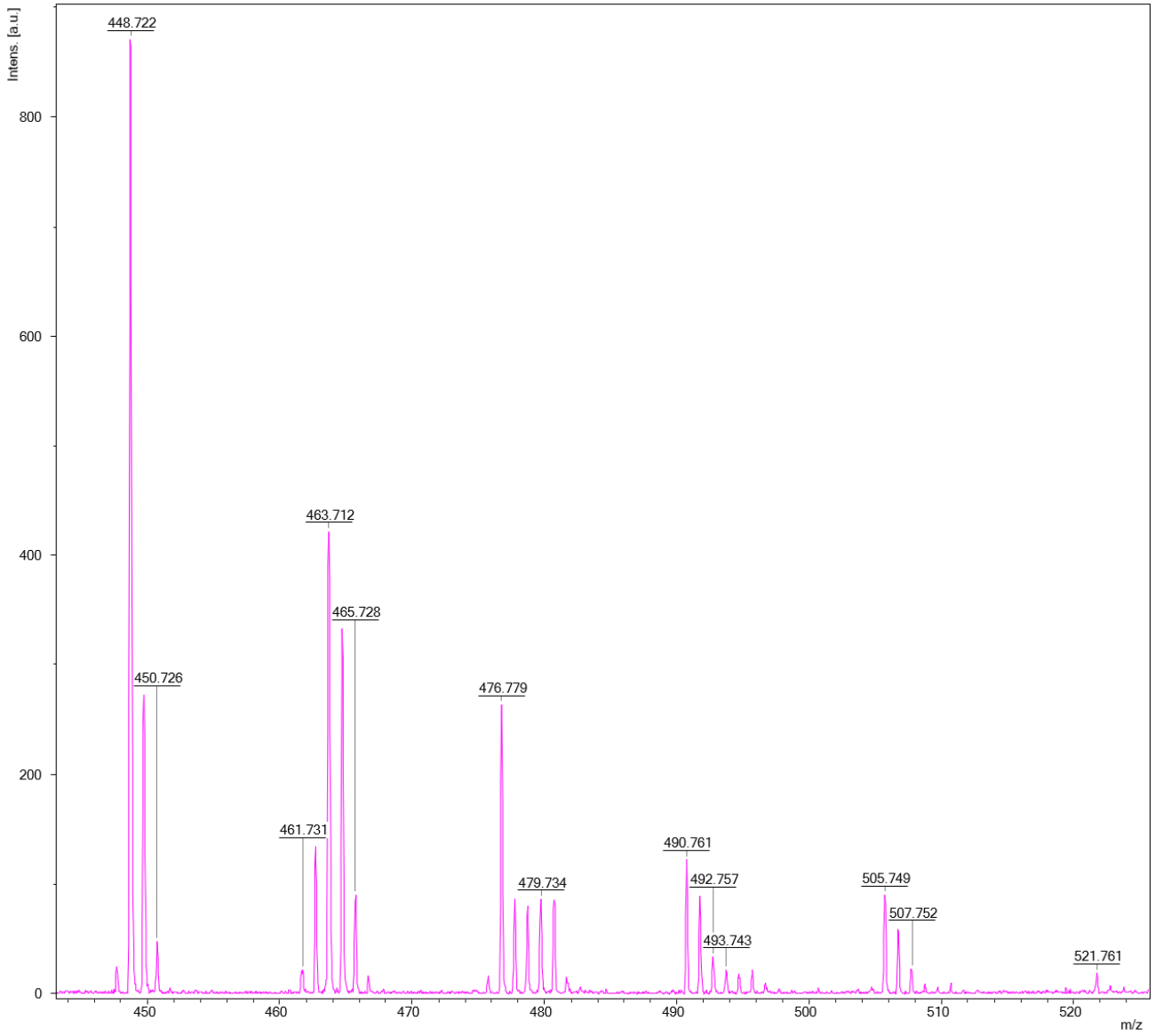
SA matrix in reflectron positive mode



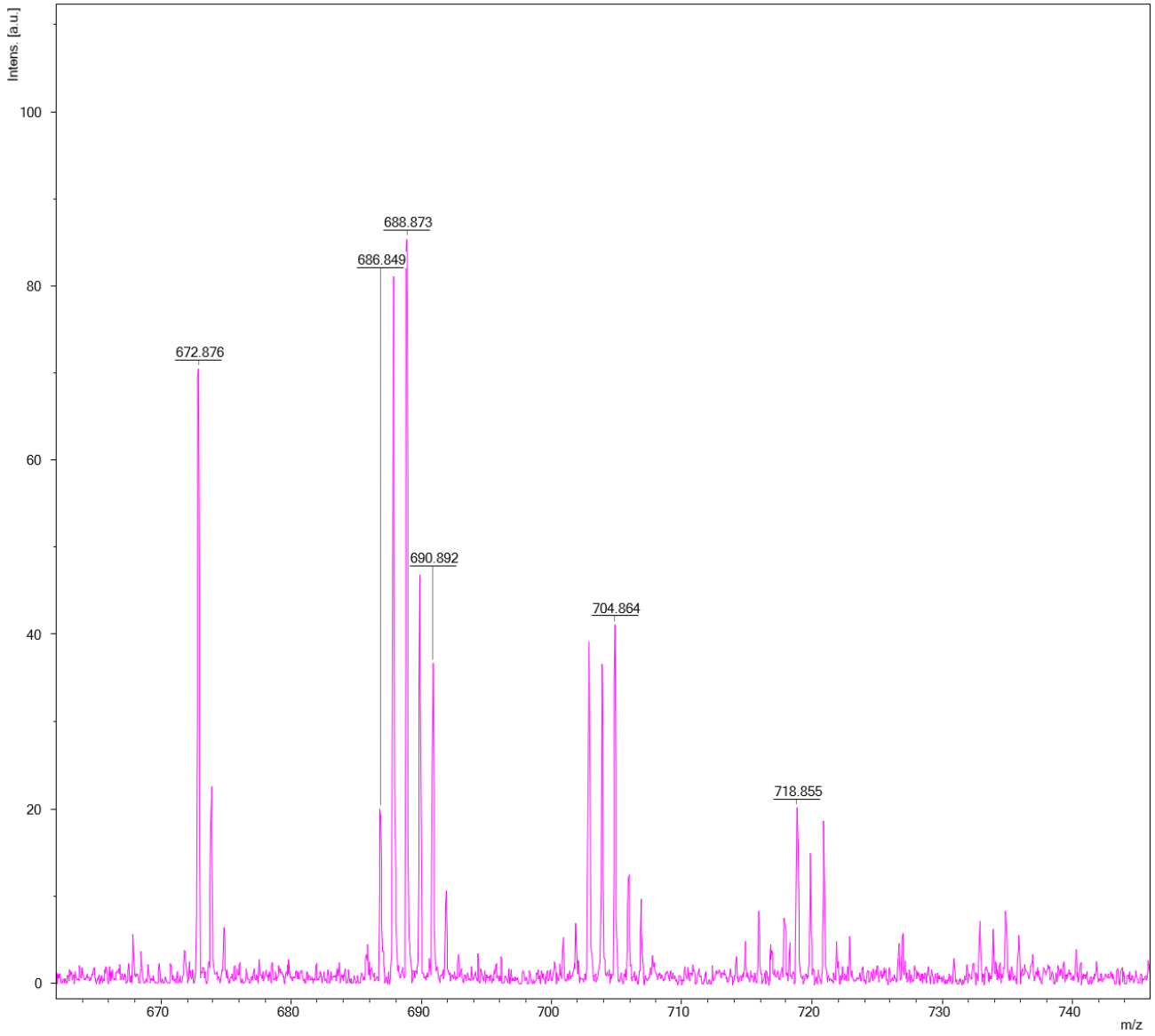
Dithranol matrix in reflectron negative mode



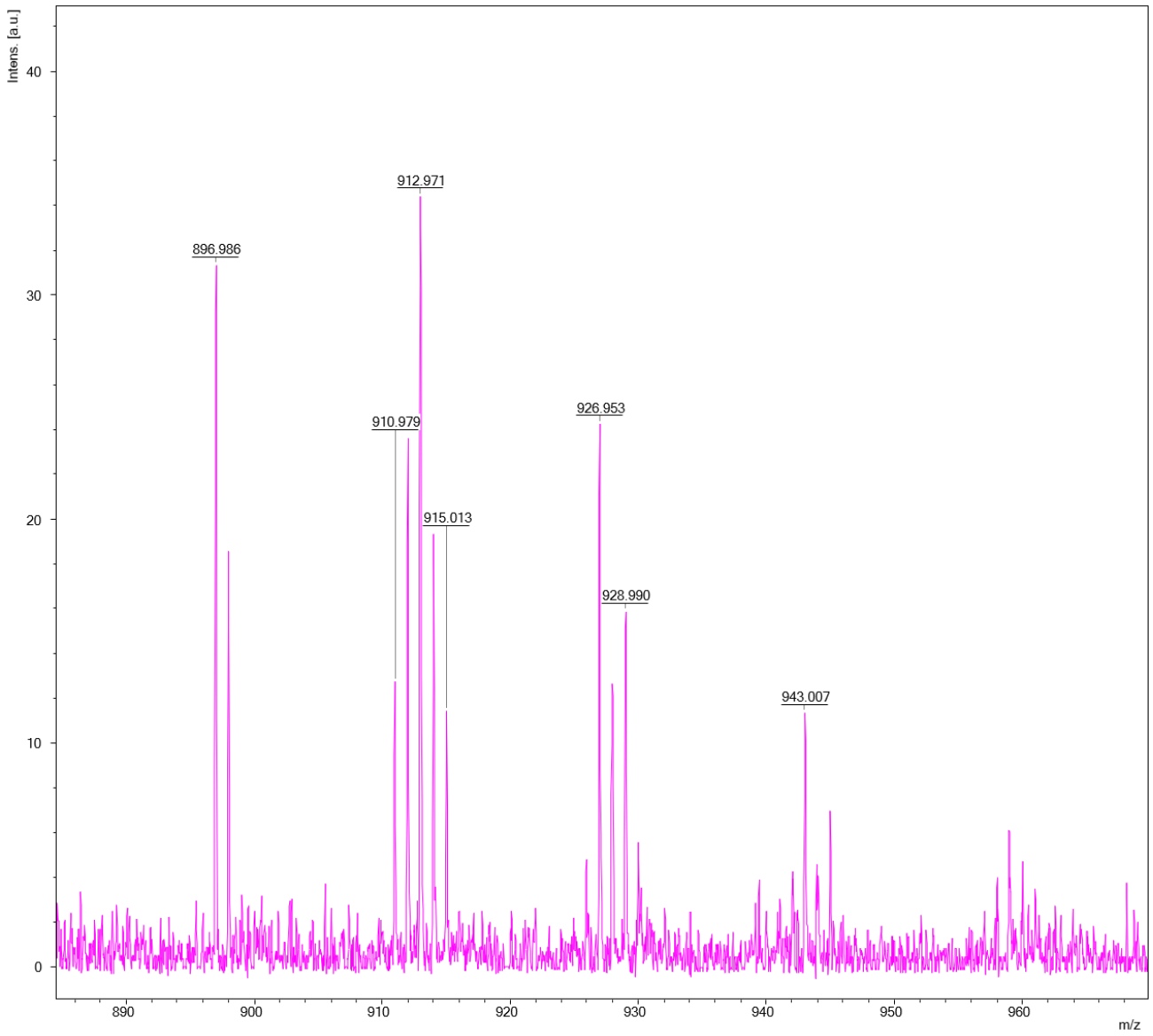
Zoom of dithranol matrix in reflectron negative mode



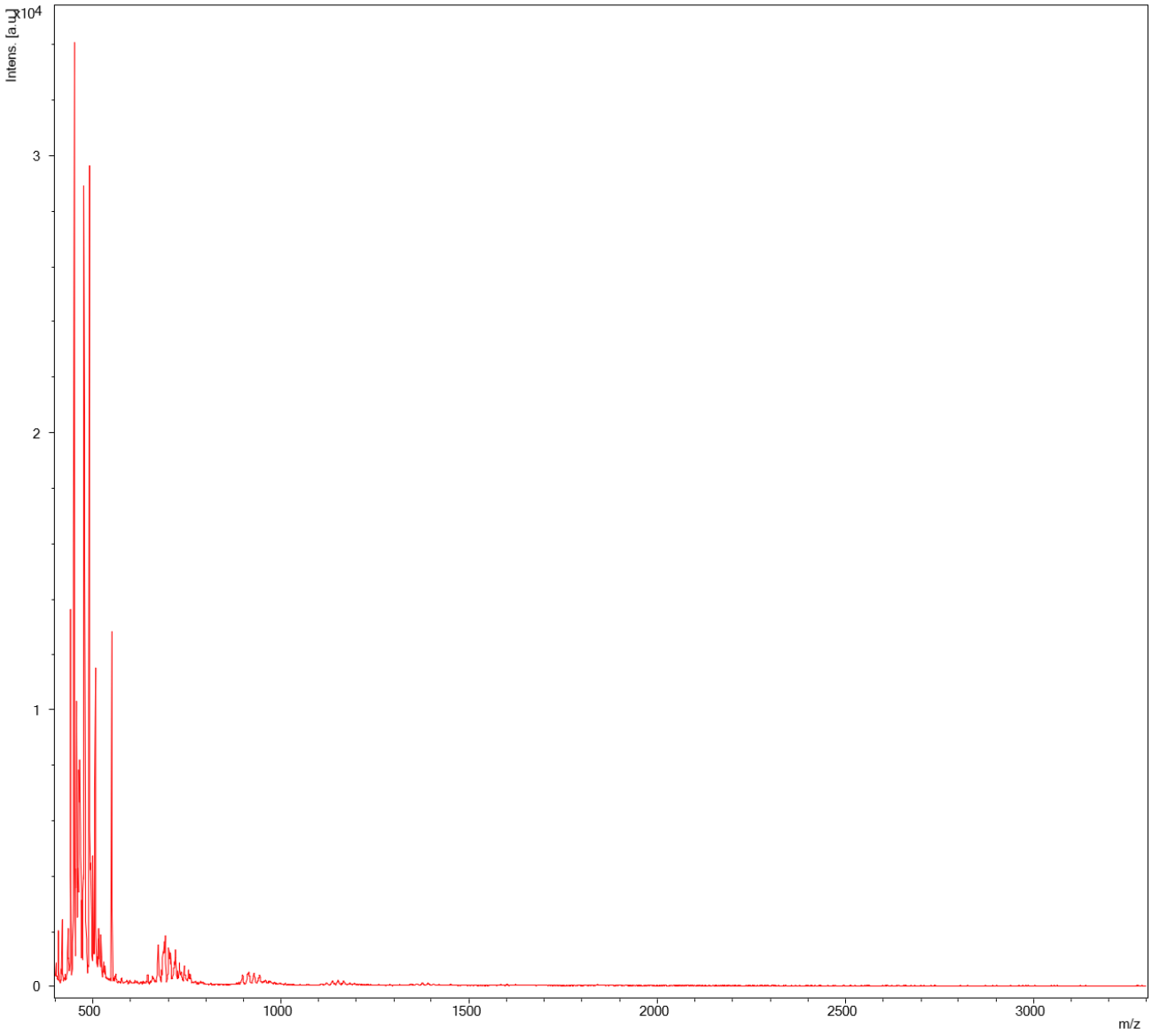
Zoom of dithranol matrix in reflectron negative mode



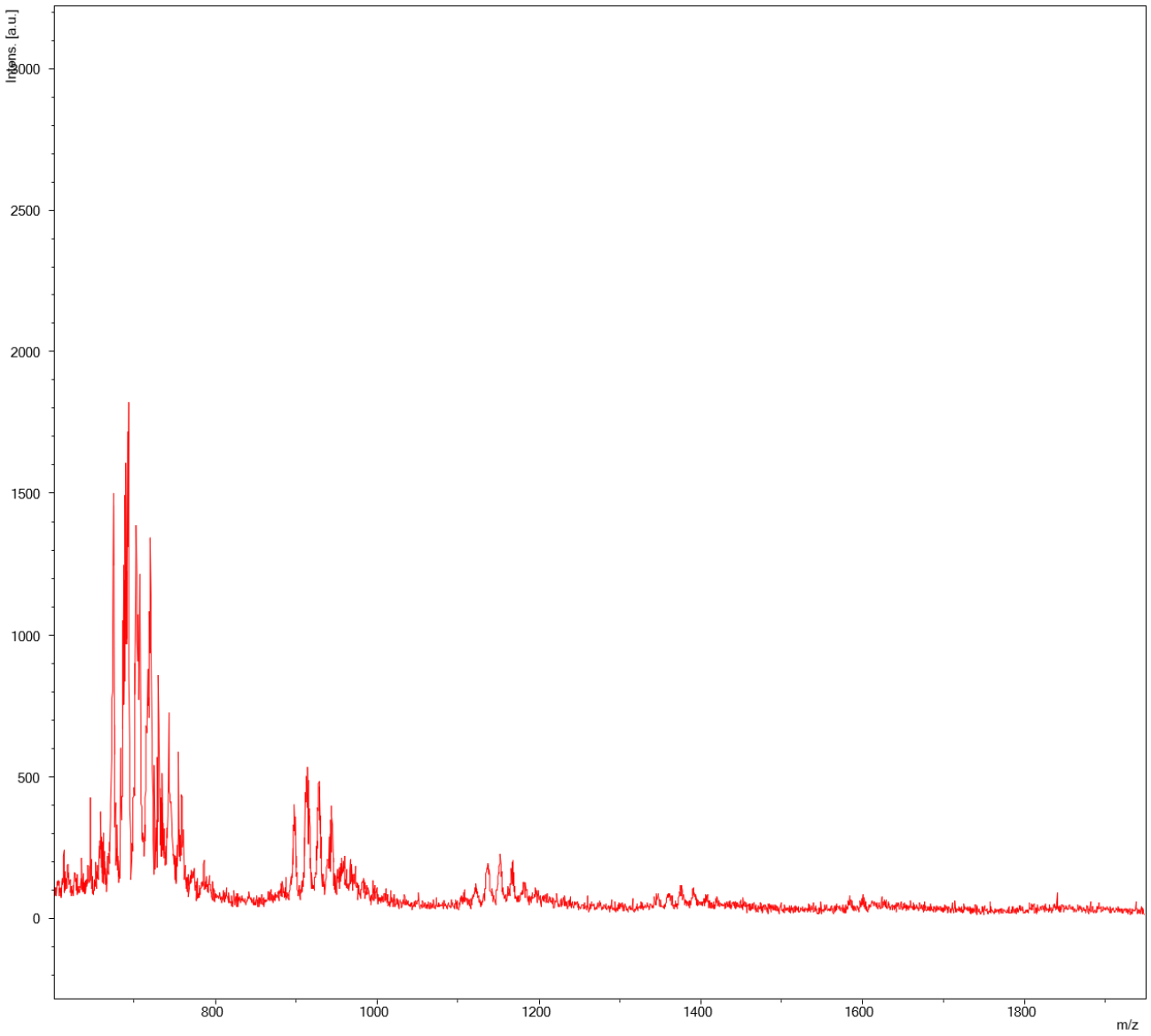
Zoom of dithranol matrix in reflectron negative mode



Zoom of dithranol matrix in reflectron negative mode



Dithranol matrix in reflectron positive mode



Zoom of dithranol matrix in reflectron positive mode