High-throughput experimentation reaction monitoring and analysis of chemistry synthesis products with ultrahigh-throughput 4D timsTOF fleX MALDI-2 technology

MALDI mass spectrometry is uniquely suited for high-throughput experimentation (HTE) chemical reaction monitoring by coupling it with trapped ion mobility spectrometry (TIMS) on Bruker's timsTOF fleX platform.

Abstract

MALDI-2, a novel ionization technique introduced recently on the timsTOF fleX, further increases accessible chemical space. TIMS provides the perfect complementary technology for HTE monitoring by MALDI by separating matrix ions and other interfering background using ion mobility. The combination of novel MALDI-2 and smartbeam 3D laser technology provides accurate, reproducible, and quantitative data for small molecules. Confident identification and confirmation of chemical synthesis products is based on 4D physical properties including collisional cross-section, accurate mass, isotope pattern, and CID-MS/MS fragmentation. The timsTOF fleX MALDI-2 method requires low sample amounts (picomoles per analysis), offers high acquisition speed (typically in the range of 1 second/sample or less), and is well suited for automation.

Keywords: timsTOF fleX, timsTOF fleX MALDI-2, drug discovery, lead discovery, lead optimization, chemical synthesis, API, API synthesis, ion mobility, CCS, HTE, high-throughput experimentation, high-throughput chemistry

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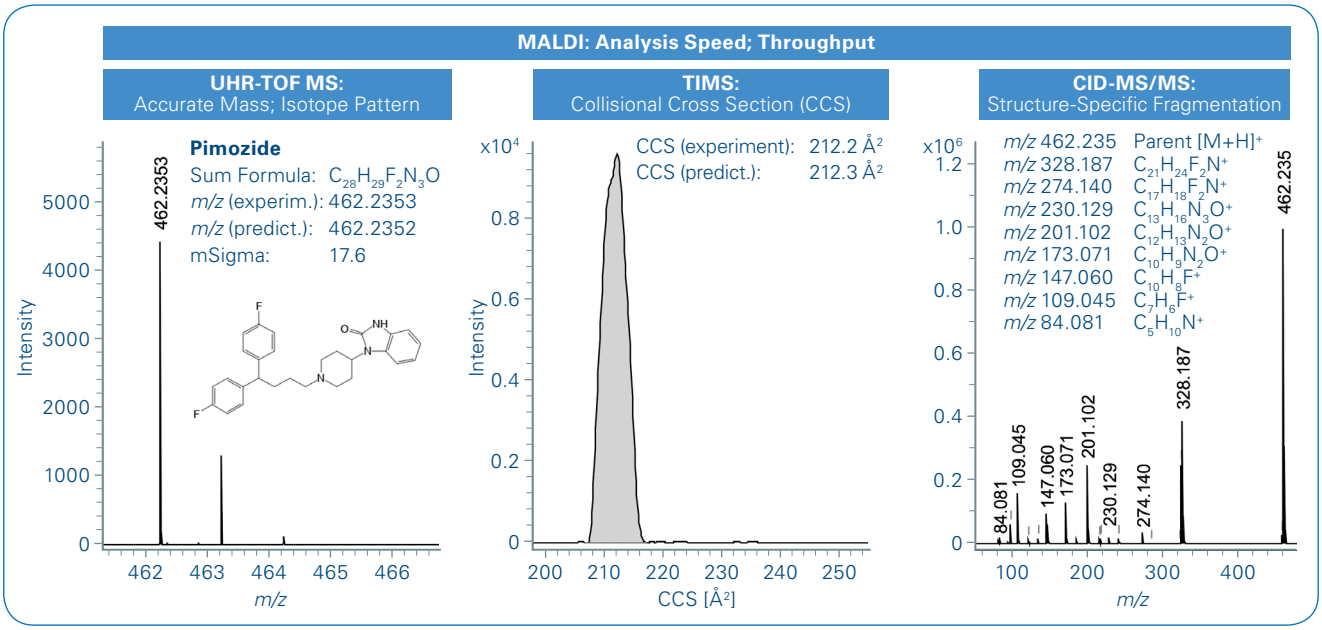

Figure 1. timsTOF fleX MALDI dataset obtained from Pimozide, an antipsychotic drug molecule.

Introduction

Structural design and chemical synthesis of new organic molecules represent important initial steps in the drug discovery process. High-throughput experimentation (HTE) is a broadly used platform for the creation of vast chemical libraries through multi-variant high-throughput chemistry. A key bottleneck in this process is the rapid analysis and confirmation of products from these reactions. Enabling faster feedback will not only reduce turnaround time for focusing on ideal reaction conditions but can also quickly reduce the number of reaction conditions that are needed, saving overall chemical costs and time. Ultrafast MALDI techniques can provide this essential near real-time data acquisition. Matrix-assisted laser desorption ionization (MALDI) mass spectrometry (MS), due to its unparalleled analysis speed, has proven itself to be well suited for pharma-related high-throughput screening applications [1-3]. The Bruker timsTOF fleX MALDI-2 instrument for the first time provides high-speed MALDI capabilities in combination with ultrahigh-resolution time-of-flight (UHR-TOF) mass spectrometry (MS) and, most critically, trapped ion mobility spectrometry (TIMS).

We introduce how timsTOF fleX MALDI-2 can be utilized for high-throughput chemical synthesis screening. The benefits of the technology include rapid and reliable identification and confirmation of drug relevant chemical synthesis products based on measuring 4D physical properties - collisional cross-section (CCS), accurate mass, isotopic pattern, and CID-MS/MS fragmentation. Because CCS is measured for all molecules, this additional property can be exploited in new ways using CCS predictive programs. By predicting CCS values for small molecules based on structure, measured outliers can be flagged for further investigation, and ideally, we can predict whether isomeric species can be separated at all.

Materials and Methods

Drug reference compounds were obtained from various commercial sources. Stock solutions in DMSO were diluted (unless otherwise specified) to a concentration of 100 μM using 50/50 (v/v) acetonitrile/water as a diluting solvent.

Microliter aliquots of 95 chemical synthesis reactions in DMSO (expected product concentration 5-50 μM) were provided by an external source in a 96 well plate. Wells were diluted 20-fold using the solvent mixture mentioned before.

0.5 μl sample aliquots were spotted on a MTP Anchorchip 384 BC MALDI plate and dried down under desiccator vacuum. Dried samples were overlayed with 0.5 μl of HCCA matrix solution (1.4 mg/mL HCCA dissolved in a mixture 90/10 (v/v) ACN/H_2O containing 0.1% TFA and 1 mM $NH_4H_2PO_4$). Matrix preparation spots were dried down at room temperature.

For MALDI-2 analyses, 2,5-DHAP was applied as a matrix following the same spotting protocol as described for HCCA, but using a matrix solution with a concentration of 2 mg/mL.

All data were acquired on a Bruker timsTOF fleX equipped with an ESI/MALDI dual ion source and MALDI-2

option. The instrument was operated in positive ion polarity automation mode controlled by timsControl 2.0 software. MALDI-MS spectra were accumulated from 4000 laser shots at 10 kHz laser repetition rate. MALDI-TIMS-MS data were acquired from 800 laser shots applying a $1/K_0$ trapping gradient 0.6 – 1.7 V*s/cm^2 and a TIMS cycle ramp time of 150 ms. For selected samples, manual analysis was performed applying customized parameters, i.e. zoomed $1/K_0$ gradient, TIMS cycle ramp time of 300 ms and a higher number of accumulated laser shots. MALDI-MS/MS spectra were acquired manually accumulating 6000 laser shots applying stepped collision energies between 20 and 120 eV.

m/z calibration was performed in MALDI mode using red phosphorous as a reference substance. Ion mobility calibration was performed in ESI mode by infusing ESI-L Low Concentration Tuning Mix.

Data were processed in DataAnalysis 5.3 software. Data processing included the following steps: peak finding including charge state assignment, matching of isotope pattern using DataAnalysis´ SmartFormula feature, generation and integration of Extracted Ion

Table 1. MALDI-TIMS-UHR-TOF MS results obtained from analysis of 30 drug reference compounds

No.	Compound Name	Sum Formula	Ion	Accurate Mass			Isotope Fit	Collisional Cross Section	
				m/z (theor.)	*m/z* (meas.)	Dev. [ppm]	mSigma	CCS (meas.) [A^2]	RSD [%]
1	Thioridazine	$C_{21}H_{26}N_2S_2$	$[M+H]^+$	371.1610	371.1612	0.6 (-1.5)*	15.9	185.4	0.03
2	Rifabutin	$C_{46}H_{62}N_4O_{11}$	$[M+H]^+$	847.4488	847.4485	-0.3	26.1	277.0	0.02
3	Desloratadine	$C_{19}H_{19}ClN_2$	$[M+H]^+$	311.1310	311.1307	-0.9	10.9	175.2	0.13
4	Clomipramine	$C_{19}H_{23}ClN_2$	$[M+H]^+$	315.1623	315.1618	-1.5	7.9	174.0	0.10
5	Chlorpromazine	$C_{17}H_{19}ClN_2S$	$[M+H]^+$	319.1030	319.1026	-1.2	7.5	170.6	0.03
6	Toremifene	$C_{26}H_{28}ClNO$	$[M+H]^+$	406.1932	406.1927	-1.1	12.8	202.2	0.08
7	Amoxapine	$C_{17}H_{16}ClN_3O$	$[M+H]^+$	314.1055	314.1052	-0.9	17.2	171.6	0.03
8	Sulfinpyrazone	$C_{23}H_{20}N_2O_3S$	$[M+H]^+$	405.1268	405.1264	-0.8	17.2	194.3	0.03
9	Irbesartan	$C_{25}H_{28}N_6O$	$[M+H]^+$	429.2398	429.2396	-0.4	6.4	200.4/205.2	0.14/0.17
10	Nefazodone	$C_{25}H_{32}ClN_5O_2$	$[M+H]^+$	470.2318	470.2313	-0.9	17.1	210.3	0.10
11	Saquinavir	$C_{38}H_{50}N_6O_5$	$[M+H]^+$	671.3916	671.3913	-0.4	5.9	259.9	0.06
12	Stanozolol	$C_{21}H_{32}N_2O$	$[M+H]^+$	329.2588	329.2584	-1.0	3.4	189.5	0.08
13	Mifepristone	$C_{29}H_{35}NO_2$	$[M+H]^+$	430.2741	430.2741	0.1 (-2.5)*	9.6	219.6	0.07
14	Loratadine	$C_{22}H_{23}ClN_2O_2$	$[M+H]^+$	383.1521	383.1520	-0.3	13.5	186.6	0.09
15	Clemastine	$C_{21}H_{26}ClNO$	$[M+H]^+$	344.1776	344.1771	-1.5	5.7	181.4	0.10
16	Atorvastatin	$C_{33}H_{35}FN_2O_5$	$[M+H]^+$	559.2603	559.2603	0.1 (-2.5)*	20.2	228.9	0.03
17	Eletriptan	$C_{22}H_{26}N_2O_2S$	$[M+H]^+$	383.1788	383.1786	-0.5	15.9	185.6	0.03
18	Donepezil	$C_{24}H_{29}NO_3$	$[M+H]^+$	380.2220	380.2217	-0.8	15.1	195.2/202.0	0.08/0.00
19	Olmesartan medoxomil	$C_{29}H_{30}N_6O_6$	$[M+H]^+$	559.2300	559.2299	-0.1	5.4	220.9/229.3	0.05/ .09
20	Gefitinib	$C_{22}H_{24}ClFN_4O_3$	$[M+H]^+$	447.1594	447.1594	0.1	8.8	200.6/206.2/ 210.7	0.03/0.13/ 0.05
21	Atazanavir	$C_{38}H_{52}N_6O_7$	$[M+H]^+$	705.3970	705.3967	-0.3	4.0	257.7	0.04
22	Haloperidol	$C_{21}H_{23}ClFNO_2$	$[M+H]^+$	376.1474	376.1471	-0.6	11.0	193.6	0.06
23	Verapamil	$C_{27}H_{38}N_2O_4$	$[M+H]^+$	455.2905	455.2903	-0.5	5.4	205.5/209.0	0.06/0.05
24	Pimozide	$C_{28}H_{29}F_2N_3O$	$[M+H]^+$	462.2352	462.2352	0.0	18.5	212.3	0.03
25	Flecainide	$C_{17}H_{20}F_6N_2O_3$	$[M+H]^+$	415.1451	415.1451	0.0	15.2	192.3	0.05
26	Zafirlukast	$C_{31}H_{33}N_3O_6S$	$[M+Na]^+$ $[M.]^+$	598.1982 575.2085	598.1978 575.2089	-0.7 0.8 (-2.5)*	17.8 12.1	233.8	0.07
27	Disopyramide	$C_{21}H_{29}N_3O$	$[M+H]^+$	340.2384	340.2381	-0.9	10.6	179.6	0.12
28	Propafenone	$C_{21}H_{27}NO_3$	$[M+H]^+$	342.2064	342.2063	-0.2	40.6	177.1	0.16
29	Imatinib	$C_{29}H_{31}N_7O$	$[M+H]^+$	494.2663	494.2663	0.0	30.1 (155.7)*	212.6	0.03
30	Ezetimibe	$C_{24}H_{21}F_2NO_3$	$[M-H_2O+H]^+$ $[M+H]^+$	392.1457 410.1562	392.1454 410.1558	-0.7 -0.8	3.1 7.6	200.2 192.2 / 198.4	0.13 0.03 / 0.05

All data represent mean values from n=3 replicate MALDI spots. Data labeled with asterisk (*) indicate critical cases where MALDI-TIMS-MS successfully resolved isobaric interferences that had affected mass accuracy or isotopic fit in MALDI-MS analysis (MALDI-MS: grey colored values in clambs).

Mobilograms of target *m/z* features. For quantitative analysis, a customized DataAnalysis script was used for extraction of target *m/z* attributes peak area and peak intensity from batches of MALDI-MS spectra into a *.csv formatted output file.

Predicted CCS values were retrieved from a public source (http://allccs.zhulab.cn/).

Results and Discussion

Figure 1 shows an example dataset obtained from Pimozide, an antipsychotic drug of the diphenylbutylpiperidine class. Based on multiple molecular attributes, (accurate mass, isotope pattern, collisional cross-section, and CID-MS/MS fragmentation) timsTOF fleX data provides confirmation of drug-like chemical synthesis products with a high level of confidence.

Table 1 presents compiled MALDI-TIMS-UHR-TOF MS results obtained in a proof-of-concept experiment including a panel of 30 commercial drug substances. Data acquisition in MALDI-MS mode took less than 1 second per sample. In MALDI-TIMS-MS mode, additional time required for TIMS separation is typically in the range of 100-300 milliseconds per frame (frame: a single ion mobility separation cycle separating MALDI ions generated and trapped from a given number of laser shots). Accuracy of the MALDI-UHR-TOF MS data was in the range of 1 ppm or better. At the same time, mSigma values (a value assigned comparing predicted isotopic pattern vs. measured, lower is better) were excellent, indicating outstanding isotopic fidelity of the data. For a few

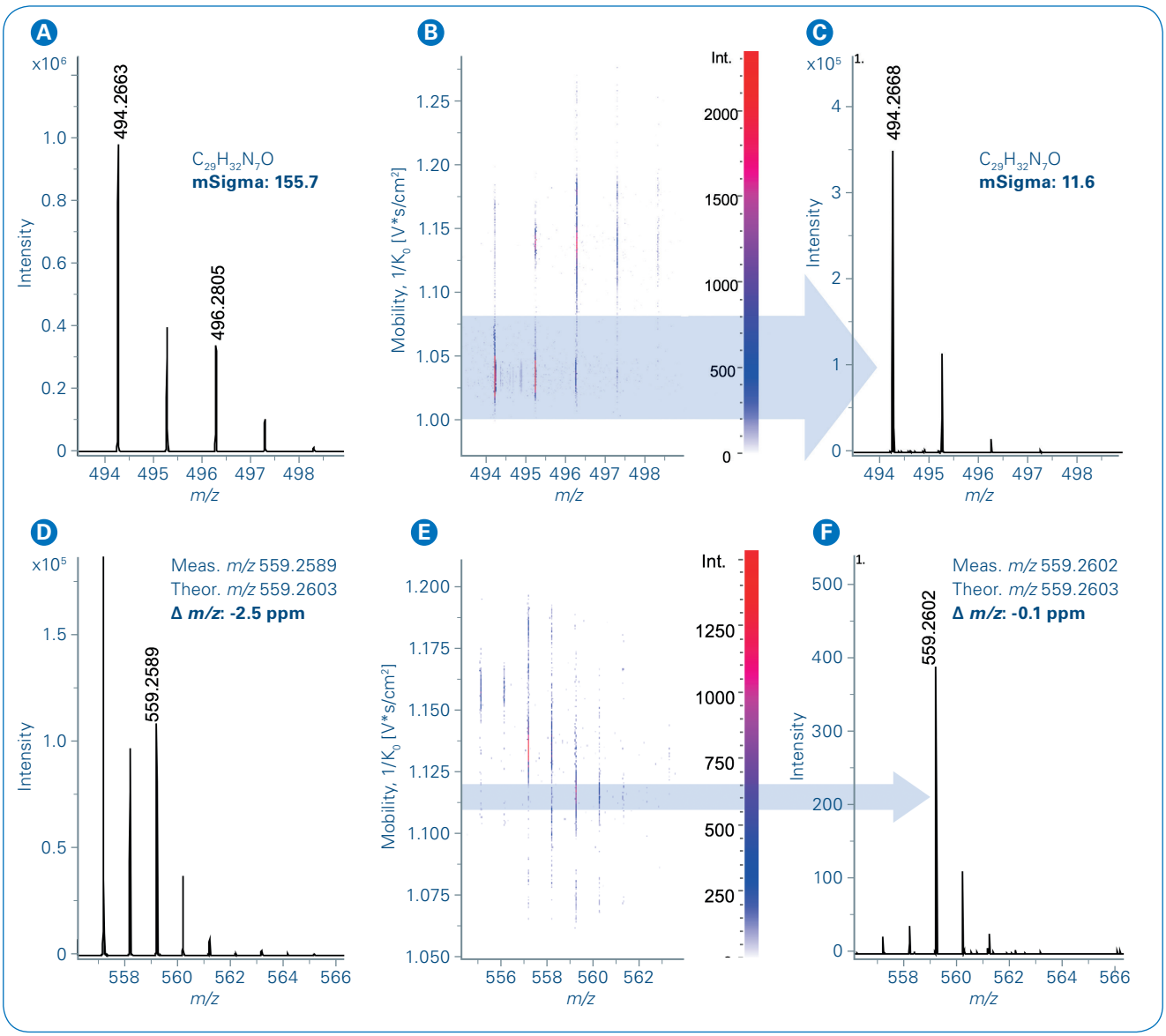

Figure 2. Example data demonstrating the benefit of TIMS as an ultrafast separation method orthogonal to MS, resolving isobaric interferences.
(A)-(C): Imatinib; (D)-(F): Atorvastatin.

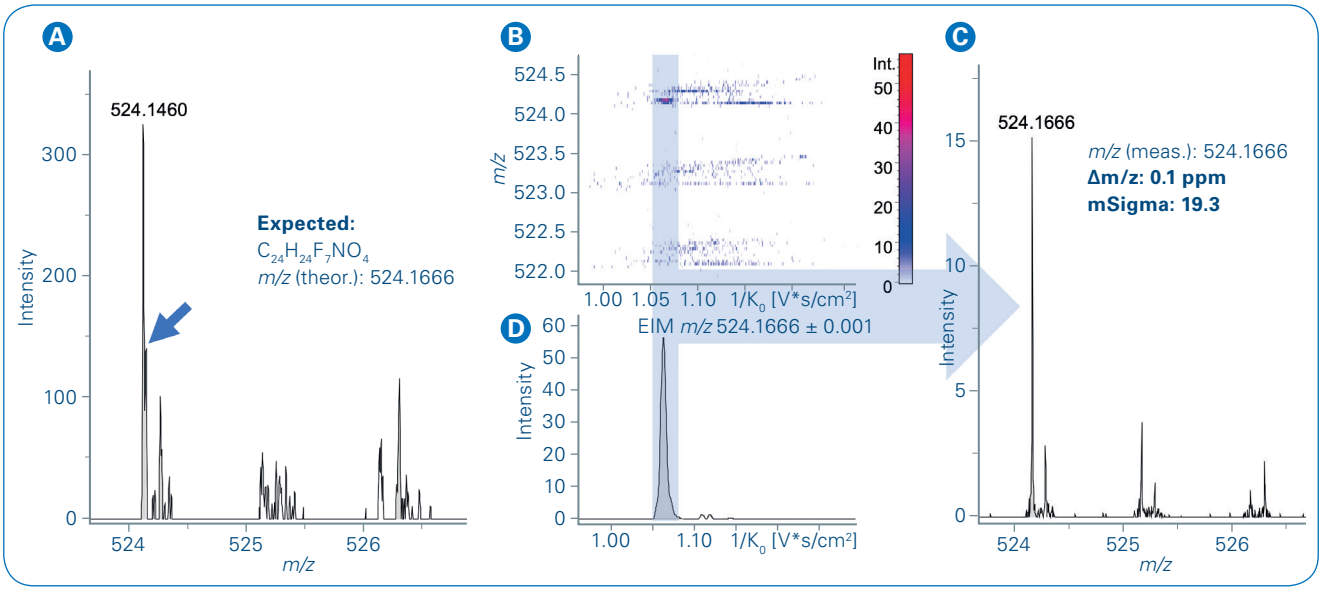

Figure 3. MALDI-TIMS-MS analysis of a chemical synthesis reaction mixture. The expected product compound (sum formula $C_{24}H_{24}F_7NO_4$; structure not disclosed; calculated $[M+H]^+$ m/z 524.1666) was barely detectable in MALDI-MS (A) due to overlap with a background signal of higher abundance. Engaging MALDI-TIMS-MS enabled efficient separation of isobaric interferences (B)-(C) allowing for unambiguous confirmation of the expected reaction product (D).

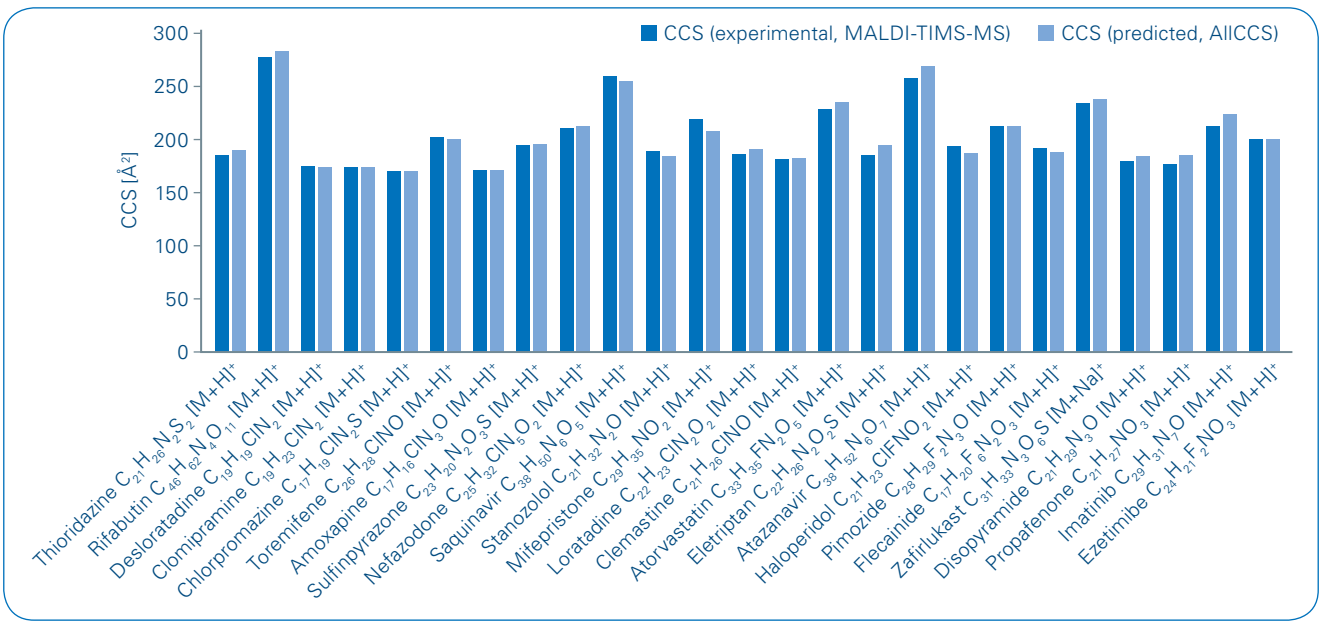

Figure 4. MALDI-TIMS-MS derived CCS values of various drug compounds matched against predicted CCS values (source: http://allccs.zhulab.cn).

compounds, significant deviations from expected mass or isotope pattern were observed, likely caused by isobaric interferences. Engaging MALDI-TIMS-MS, these overlaps were resolved enabling virtually interference-free spectra and confident confirmation of compound identity (Figure 2).

In a second experiment, microliter aliquots of 95 chemical synthesis reactions were analyzed. For 88 out of 95 reactions (93%), the expected product was successfully detected at low to sub-ppm mass accuracy (≤1 ppm: 74; 1-2 ppm: 16; 2-3 ppm: 3). In critical cases, TIMS enabled efficient separation of the expected product compound from interfering background resulting in unambiguous identification of the expected synthesis product. An example is shown in Figure 3.

For the panel of 30 commercial drug compounds, CCS values were calculated from calibrated MALDI-TIMS-MS data (see Table 1). RSD of these CCS values (n=3), on average, was 0.07% confirming outstanding reproducibility of the underlying MALDI-TIMS-MS data. Experimental CCS values were matched against predicted CCS values derived from machine learning algorithms (source: http://

allccs.zhulab.cn). For most compounds, experimental and predicted data were in good agreement with each other, with deviations ranging from 0.1 to 5.3% (average: 2.2%). Matching results presented in Figure 4 indicate promising potential of prediction-based approaches for CCS aware ID confirmation of newly synthesized drug molecules, for which experimental reference data might not be available.

To demonstrate quantitation capabilities, 3 reference compounds (perphenazine, sulfadimethoxine, sulfaguanidine) were analyzed in mixture using verapamil as an internal standard at 1 nM final

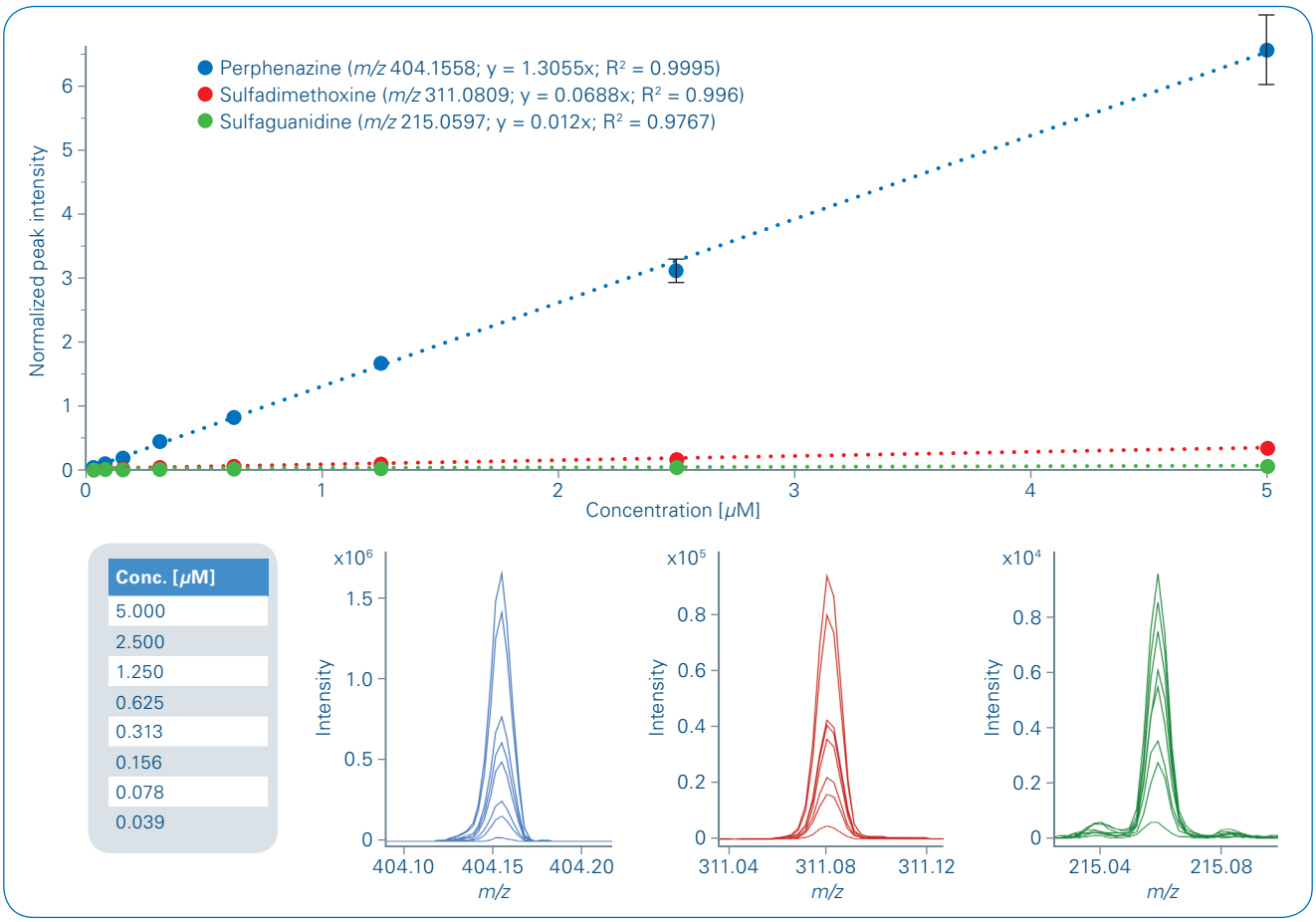

Figure 5. Quantitative MALDI-MS analysis of three selected drug reference compounds in mixture using verapamil as an internal standard.

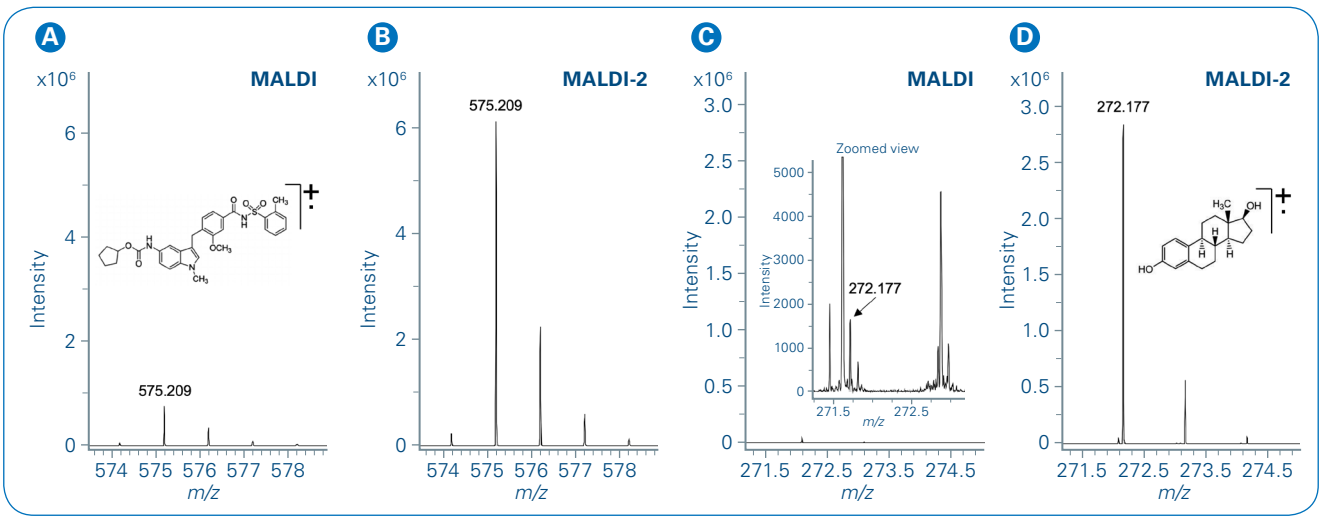

Figure 6. Enhanced ionization of zafirlukast (Ⓐ-Ⓑ) and estradiol (Ⓒ-Ⓓ) by laser post-ionization based MALDI-2

concentration (Figure 5). All three compounds, after normalization against the internal standard signal, yielded linear response curves over at least two orders of magnitude (0.039 – 5 μM).

Laser post-ionization based MALDI-2 technology provides access to an expanded analyte space by enhancing ionization of molecules that have been out of scope of classical MALDI [4]. This is demonstrated in Figure 6. A more than 5-fold increase in signal intensity was detected in MALDI-2 for the radical molecular ion M.+ of leukotriene receptor antagonist zafirlukast ($C_{31}H_{33}N_3O_6S$, Figure 6 A-B). Even stronger MALDI-2 response was observed for certain steroid hormones, i.e. estradiol (Figure 6 C-D).

Conclusion

- High-throughput experimentation can be accelerated with higher efficiency by Bruker's 4D timsTOF fleX with MALDI-2 technology. timsTOF fleX with MALDI-2 is ideal for high-throughput analysis of chemical synthesis products in drug discovery, measuring reproducible collisional cross-sections, accurate mass, true isotope pattern, and CID-MS/MS fragmentation data.
- TIMS provides a critical benefit as an ultrafast separation principle reducing or eliminating matrix and other isobaric interferences. It also yields highly reproducible CCS values as an additional physical property, enhancing confirmation of identity of newly synthesized molecules.
- MALDI-2 provides access to an expanded analyte space by enhancing ionization of compounds that were out of scope of conventional MALDI.
- The timsTOF fleX MALDI-2 method offers high analysis speed (typically in the range of 1s per sample or less), requires low sample amounts (picomoles per analysis spot) and is compatible with a high level of automation by using liquid handlers for MALDI preparation.

timsTOF fleX MALDI-2 key technology feature	Customer benefit for pharma related applications
Dual MALDI / ESI ion source	Instant switching between MALDI and ESI ionization modes within seconds; enabling MALDI guided SpatialOMx® by integrating MALDI Imaging with LC based 4D-Omics
10 kHz smartbeam 3D laser technology	Ultrahigh MALDI acquisition speed & throughput; Spatial definition in TruePixel MALDI imaging
MALDI-2 laser post-ionization	Expanded analyte space providing access to molecules not ionizable in conventional MALDI
Trapped Ion Mobility Spectrometry (TIMS)	Additional dimension of ultrafast separation orthogonal to MS; separation of isobars and isomers; highly reproducible Collisional Cross-Section (CCS) values enabling CCS aware compound identification and 4D-Omics
Parallel Accumulation Serial Fragmentation (PASEF®)	Enabling 4D-Omics at ultimate speed, depth and sensitivity
Ultrahigh Resolution TOF (UHR-TOF)	Full-sensitivity resolution (FSR); sub-ppm mass accuracy; ultimate dynamic range; True Isotopic Pattern (TIP)

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