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Achieving Highly Accurate CCS Measurements in LC-IM-MS Analyses

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Introduction

Recent published studies¹ have evaluated the inter-laboratory reproducibility of CCS measurements with results as low as 0.3% using the stepped field method on low field drift tube ion mobility MS systems. The work also reported values achieved under controlled and traceable instrumental conditions, and based on the Mason-Schamp equation provided reference values for both calibration standards and many analytes. Based on these reference values and single field calibration 0.6% (%RSD) variation in CCS values was observed using direct sample infusion. The results presented in this work advance previously reported methodologies towards highly accurate CCS measurements for LC-MS analysis and establishes limits that can be set for CCS as a molecular identifier.

Experimental

Laboratories

Experimentation was performed in three separate laboratories: Agilent, (Santa Clara, CA) Univ. Duisburg-Essen (Essen, Germany) and BOKU (Vienna, Austria).

Instrumentation

Experiments were performed on commercial LC (1290 Series) – Ion Mobility Mass Spectrometer (6560 Ion Mobility Q-TOF with Alternative Gas Kit, Agilent Technologies, Santa Clara, CA)

Samples

Avanti Lipidomix standard (1 mg/mL) from Avanti Polar Lipids (Alabaster, AL; Sigma, Germany) was diluted 5 µl into 1.5 mL 1:1 chloroform:methanol to prepare a nominal 5 µM concentration (mw=650).

Agilent Tunemix (G1969-85000) was diluted 1:10 in 95:5 acetonitrile:water. Agilent Reference Mass Std – 50 µl of Purine (5 µM) and 10 µl of 921 (2.5 µM) were diluted into 95:5 acetonitrile:water.

Reference Mass Introduction

For recalibration of the mass axis, the reference mass solution was introduced via a Tee connection into the LC effluent just ahead of the Agilent JetStream source with a flowrate of 10 µl/min to maximize vaporization of the acetonitrile in the reference solutions.

Standard Source Conditions

Drying Gas Temperature: 300°C, Drying Gas Flow Rate: 9 L/min, Nebulizer Pressure 20psi, Vcap: 4000V, AJS Nozzle: 2000V, Sheath Gas 12 L/min, Sheath Gas Temp 275°C

Experimental

Ion Mobility Conditions

Settings: the Trap Fill Time to 20 msec and 250 µsec for Trap Release Time.

Stepped Field Method: 7 steps as published with field varied from 10.9 V/cm to 18.5 V/cm

Single Field Method: Equal to the 6th step of the Stepped Field Method – approx. 17.3 V/cm.

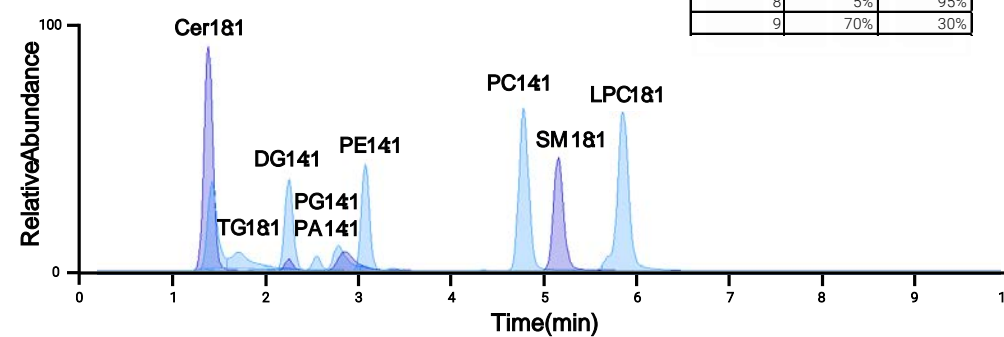
MS Method

Autotuned in 1700 mass range mode, then set Ion Funnel RF setpoints to 120 V.

LC Method

- RX-SIL Column (3.0mm x 100mm x 1.8 micron)
- 40°C – column temp, std flow rate = 0.36 mL/min
- Solvent A: ACN with 0.2% formic acid
- Solvent B: ACN:MeOH:H₂O (50:20:30 v/v) with 20mM ammonium formate
- Gradient Elution Program
- Lipid Standards Chromatogram

Min.	Solvent A	Solvent B
0	70%	30%
2	40%	60%
4	30%	70%
5	5%	95%
8	5%	95%
9	70%	30%



Varied Analytical Conditions

The Source Drying Gas temperature was evaluated at 250, 300 and 350°C. The Drying Gas flow was evaluated at 7 L/min, 9 L/min and 12 L/min. The HPLC flow was evaluated at 260 µl/min, 360 µl/min and 460 µl/min.

Software

Acquisition software was MassHunter QTOF Acquisition B.08. MS IRM calibration was made using IMS Reprocessor. Stepped and Single Field CCS Calibrations were made using B.08 IM-MS Browser. Feature Detection and Identification were made using B.08 Mass Profiler and ID Browser.

Results and Discussion

The principle focus of this study is to investigate intra-lab repeatability and inter-lab agreement with regards to CCS measurement as applied to a set of lipid standards and thereby determine confidence limits that can be used as part of multidimensional database searching.

Creating Stepped Field CCS Databases

The 6560 IM-QTOF with its low field drift tube ion mobility analyzer and adherence to the Mason-Schamp equation is ideal for use in producing accurate CCS values. The "Stepped-Field" method increases the axial potential across the drift tube and records the corresponding ion arrival time. The extrapolation of 1/axial potential gives an intercept equivalent to zero time in the drift tube and thus the time outside the drift tube. Subtracting the time spent outside the drift tube from the observed total time results in the time to traverse the drift tube and using the Mason-Schamp equation, this drift time can be used to determine CCS values. Typical CCS variation across the 7 field steps is within 0.15% for Agilent Tunemix and 0.25% for the lipid standard ions.

Ion	Agilent	DE	BOKU	Ave.	%SD	Ref.*	% Delta
118	121.28	121.59	121.44	121.43	0.13%	121.30	0.11%
322	153.60	153.70	153.59	153.63	0.04%	153.73	-0.06%
622	202.94	203.49	202.58	203.00	0.22%	202.96	0.02%
922	243.40	243.78	242.91	243.36	0.18%	243.64	-0.11%
1222	282.17	282.65	281.08	281.97	0.29%	282.20	-0.08%
1522	317.10	317.49	315.63	316.74	0.31%	316.96	-0.07%

*Reference values established in InterLab Study¹

Values are average of triplicate measurements Mean -0.03%
SD 0.08%

Lipid	Adduct	Agilent	DE	BOKU	%SD
Cer 18:1	[M+H] ⁺	256.81	256.12	256.19	0.15%
Cer 18:1	[M+Na] ⁺	255.40	254.87	254.49	0.18%
DG(14:1/14:1)	[M+H-H ₂ O] ⁺	234.75	234.45	234.12	0.14%
DG(14:1/14:1)	[M+Na] ⁺	229.70	229.59	229.45	0.05%
LPC 18:1	[M+H] ⁺	231.66	231.29	231.15	0.11%
LPC 18:1	[M+Na] ⁺	234.87	234.59	234.29	0.12%
PA(14:1/14:1)	[M+Na] ⁺	242.32	241.54	241.50	0.19%
PC(14:1_14:1)	[M+H] ⁺	264.79	264.18	263.70	0.21%
PC(14:1_14:1)	[M+Na] ⁺	267.18	266.64	266.04	0.21%
PE(14:1/14:1)*	[M+H] ⁺	249.96	249.25	248.03	0.39%
PE(14:1/14:1)	[M+Na] ⁺	256.02	253.14	255.27	0.58%
PG(14:1/14:1)-1*	[M+Na] ⁺	250.25	249.35	249.51	0.19%
PG(14:1/14:1)-2*	[M+Na] ⁺	260.14	259.55	259.79	0.11%
SM 18:1	[M+H] ⁺	286.00	285.27	284.57	0.25%
SM 18:1	[M+Na] ⁺	286.74	286.01	285.34	0.24%
TG(18:1/18:1/18:1)	[M+Na] ⁺	324.26	323.84	323.43	0.13%
TG(18:1/18:1/18:1)	[M+NH ₄] ⁺	324.91	323.44	322.49	0.37%

* Indicates conformers observed (PE - not resolved, PG - resolved)

Values are average of triplicate measurements Mean 0.21%
SD 0.13%

The mean bias of measured Tunemix CCS values to published¹ values is 0.03%. The mean %RSD for lipid CCS values measured is 0.21%.

Evaluating Single Field CCS (Infusion) Agreement to Stepped Field Database Values

For LC separations instead of stepping the axial drift cell potential, a fixed voltage is used. Using the total drift time, the ion and drift gas masses and the ion charge state with a calibration coefficient for all other instrumental factors held constant the 6560 Single Field calibration conforms to the Mason-Schamp equation. This together with the low thermal heating from the low field drift tube results in a calibration that is analyte, adduct and charge-

state non-specific and allows for the use of Tunemix as a CCS Single Field universal calibration standard.

We first acquired the Lipid standard under the same infusion conditions (50 μ L/min) as was used for the Stepped Field measurements. The mean bias for the average of triplicate runs to Stepped Field values is 0.09% with a SD of 0.36%. The SD is reduced to 0.13% when the previously unresolved PE conformer pair is updated with CCS values from the Single Field runs. The low bias and %RSD confirms the effectiveness of the Single Field Method for the various Lipids and adducts measured.

Lipid	Adduct	Ref*	Agilent	DE	BOKU	Ave. Bias ¹	%SD ¹	Ave. Bias ²	%SD ²
Cer 18:1	[M+H] ⁺	256.81	257.20	257.03	257.24	0.14%	0.08%	-0.03%	0.06%
Cer 18:1	[M+Na] ⁺	255.40	255.92	255.86	255.89	0.19%	0.10%	-0.02%	0.01%
DG(14:1/14:1)	[M+H-H ₂ O] ⁺	234.75	235.45	235.49	235.28	0.28%	0.14%	-0.03%	0.06%
DG(14:1/14:1)	[M+Na] ⁺	229.70	230.14	230.56	230.63	0.32%	0.19%	0.20%	0.02%
LPC 18:1	[M+H] ⁺	231.66	232.05	232.30	232.33	0.24%	0.13%	0.12%	0.01%
LPC 18:1	[M+Na] ⁺	234.87	235.08	235.26	235.18	0.13%	0.07%	0.06%	0.02%
PA(14:1/14:1)	[M+Na] ⁺	242.32	242.15	242.21	242.25	-0.05%	0.03%	0.03%	0.01%
PC(14:1_14:1)	[M+H] ⁺	264.79	265.17	264.98	264.92	0.09%	0.06%	-0.08%	0.01%
PC(14:1_14:1)	[M+Na] ⁺	267.18	267.44	267.21	267.08	0.02%	0.06%	-0.11%	0.03%
PE(14:1/14:1)-1	[M+H] ⁺	252.34	252.34	252.72	252.61	0.09%	0.08%	0.13%	0.03%
PE(14:1/14:1)-2	[M+H] ⁺	247.50	247.50	247.30	247.25	-0.06%	0.05%	-0.09%	0.01%
PE(14:1/14:1)	[M+Na] ⁺	256.02	256.22	256.26	256.28	0.09%	0.05%	0.02%	0.00%
PG(14:1/14:1)-1	[M+Na] ⁺	250.25	250.80	250.77	251.05	0.25%	0.13%	0.04%	0.08%
PG(14:1/14:1)-2	[M+Na] ⁺	260.14	260.17	260.22	260.30	0.04%	0.03%	0.04%	0.02%
SM 18:1	[M+H] ⁺	286.00	286.43	286.01	285.81	0.03%	0.09%	-0.18%	0.05%
SM 18:1	[M+Na] ⁺	286.74	286.99	286.66	286.46	-0.01%	0.08%	-0.15%	0.05%
TG(18:1/18:1/18:1)	[M+Na] ⁺	324.26	324.10	323.72	323.66	-0.13%	0.09%	-0.13%	0.01%
TG(18:1/18:1/18:1)	[M+NH ₄] ⁺	324.91	324.96	324.56	324.40	-0.08%	0.08%	-0.15%	0.03%

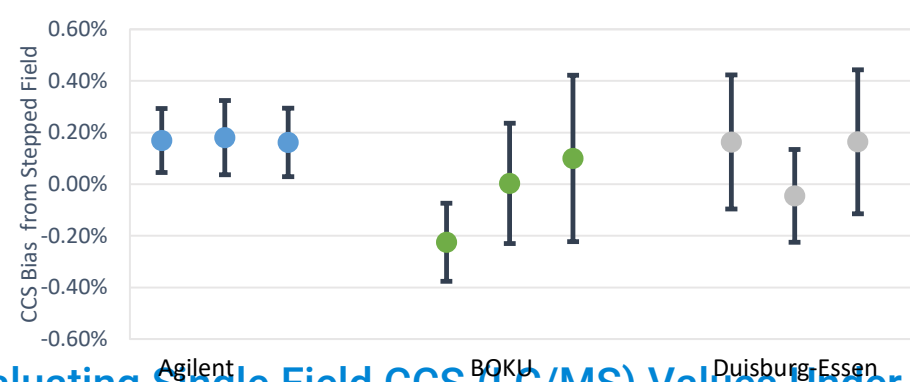
*Agilent Stepped Field Values (except PE(14:1/14:1)-1,2)

¹Agilent Stepped Field CCS as Reference values Mean 0.09% -0.02%
²Agilent Single Field CCS as Reference values SD 0.13% 0.11%

Values are average of triplicate measurements

Evaluating Single Field CCS (LC/MS) Agreement to Stepped Field Database Values

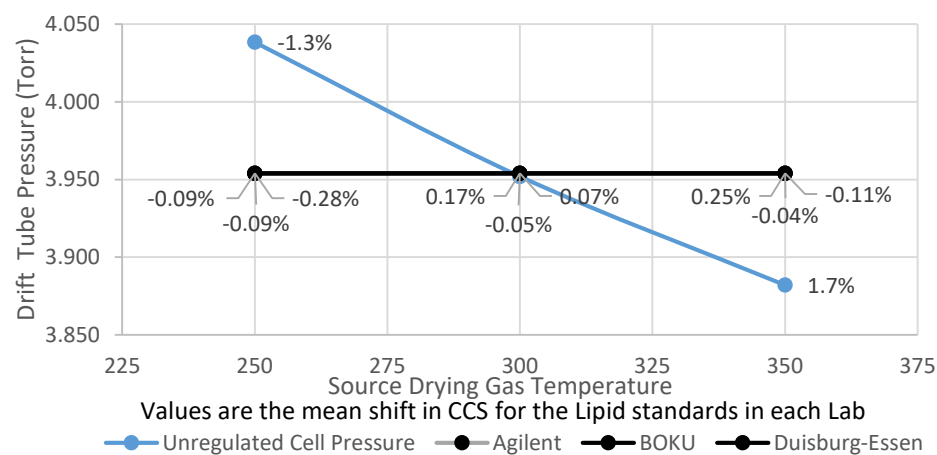
To assess the effect of moving from infusion to LC operation, the lipid panel was analyzed using a short HILIC separation under standardized conditions. The mean bias and SD for all lipids and associated adducts (obs. vs. Stepped Field CCS) was generally less than 0.20%.



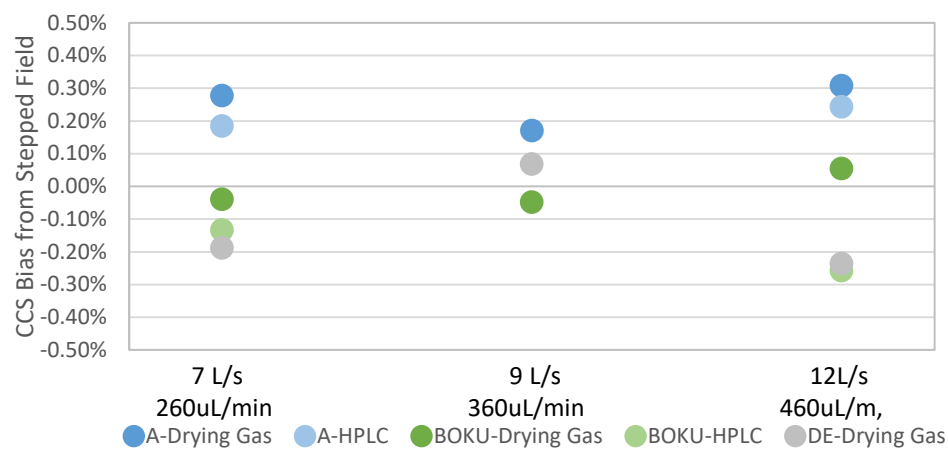
Evaluating Single Field CCS (LC/MS) Values Under Varied Source Conditions

To determine what degree altering source temperature might affect the Drift Tube pressure and CCS measurement a common Single Field calibration was applied to triplicate runs acquired source drying gas set to 250°C, 300°C and 350°C. As a comparison point data

was also acquired with Alt. Gas Kit pressure control inactivated. As is customary with API source conditions, lower temperature settings result in higher gas flow and pressure leading into the pumping stages. With the pressure control active, no measurable pressure change was observed in the Drift Tube and changes in CCS values are within normal run-to-run variation.

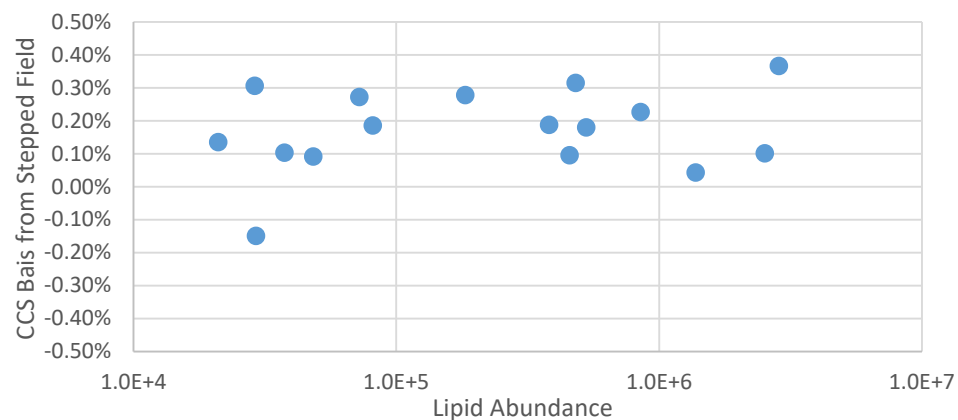


In addition to the standard 9 L/min of Drying Gas and 360 μ L/min HPLC flow rate LC/IM-MS runs were acquired at 7 L/min and 12 L/min and 260 μ L/min and 460 μ L/min solvent flow rates, also without systematic effect on CCS measurement.



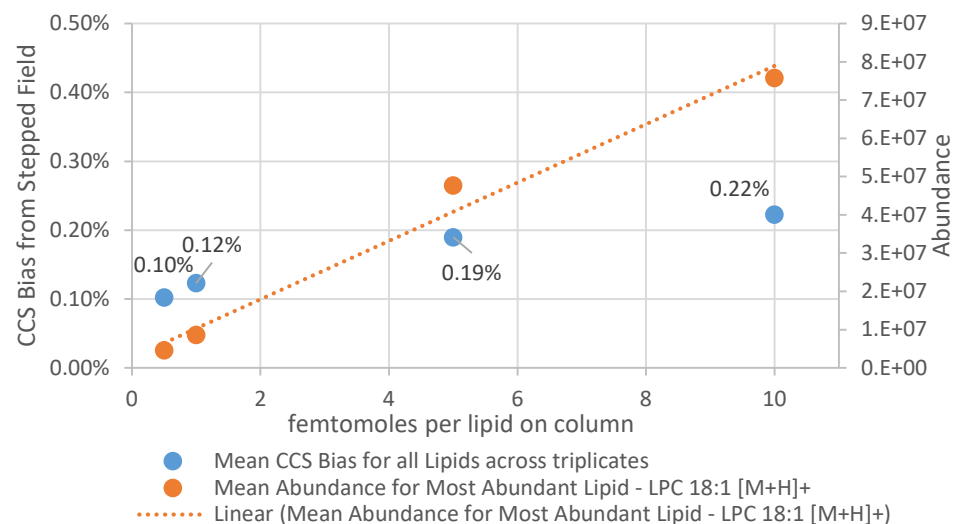
Evaluating Single Field CCS (LC/MS) Values Under Extended Dynamic Range Conditions

Allowing for the broad range in response across lipids and adducts, the flowing plot highlights variation in CCS for the Standard Conditions in the Agilent Lab showing



Approximately +/- 0.1% over 2 decades. To evaluate CCS accuracy at higher sample levels 4 bit multiplexing was

enabled by multiple shorter ion gate releases based on a pseudo random sequence and achieving a duty cycle of nearly 50%. This resulted in nearly 1 additional



decade in high end dynamic range while maintaining very close agreement to the Stepped Field database values.

Evaluating Mass Accuracy

Typical mass accuracy of less than 2 - 3 ppm was achieved though reference mass correction using m/z 121 and 922 and the IMS Reprocessor program and by offsetting the reference mass targets by 5 ppm (2 ppm for multiplexed) to account for abundance related shifts of the calibrant ions. Feature detection of the analytes intrinsically corrects for abundance related mass shifts.

Conclusions

Highly accurate CCS values determined by the standard Stepped Field method for compound databases show:

- Excellent (typically better than 0.5%) agreement to Single Field measurements using Tunemix as the calibrant
- Applicability to CCS measurements made under a broad range LC-IM/MS conditions
- Are compatible with data generated using Multiplexed Ion Mobility mode extended dynamic range
- Together with low ppm mass accuracy provide excellent specificity in support of analyte conformation.

References

- ¹An Interlaboratory Evaluation of Drift Tube Ion Mobility Collision Cross Section Measurements Stow, T. J. Causon, X. Zheng, R. Kurulugama, T. Mairinger, J. C. May, E. E. Rennie, E. Baker, R. Smith, J. A. McLean, S. Hann and F. J. Ojstedt. *Anal. Chem.* 2017, 89(17), 9065.
- ²Unraveling the Complexity of Lipidomes by Multiple Heart Cutting LC/MS with the Agilent 1290 Infinity 2 LC Solution. Vanhoenacker, R. t'Kindt, F. David, P. Sandra, and K. Sandra, Application Note: Biopharmaceuticals and Biosimilars, 2015.

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