Supplementary Information:

Separating *O*-desmethylvenlafaxine and Tramadol Enantiomers using Two-dimensional Chiral LC×DMS Mass Spectrometry

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Description of enhanced product ion scan validation experiment:

As discussed in Section 3.1 of the manuscript, enhanced product ion (EPI) scan mode available on the SCIEX QTRAP 5500 was utilized to monitor fragments of ODV and TRA at various collision energy (CE) settings. In Figure S3, the average EPI scan from CE 5 —180 V demonstrates the possibilities of differences between product ions produced by ODV and TRA. These fragments are notably present in Figure S2, but without the linear ion trap, the response has insufficient signal (within the expected concentration of ODV and TRA in wastewater influent) to be monitored. However, if unique fragments of each isomer are produced in EPI, those fragments have a potential to be selectively monitored.

Using LC Method A, 500 ng mL^{-1} standards were injected independently to determine retention times of each isomer. Then, a 500 ng mL^{-1} 1:1 mixture of ODV and TRA was injected using the same LC method, with five separate EPI experiments at different CEs (see Table S3). This experiment selected for the parent *m/z* 264 and held ions within the LIT for 3 ms. Since each EPI scan provides the fragments produced at a given retention time, product ions were monitored to assess common or unique fragments.

Description of two-dimensional chiral LC×DMS-MS^2 heatmap and CV verification:

The centroid of each peak was determined using the below methodology prior to LC×DMS-MS^2 analysis. This verification was done initially using t-infusion of the mobile phase composition and a mixture of ODV and TRA standards. This verification is also critical to ensure that shifts in optimal CV values are correctly selected for the highest sensitivity prior to daily analysis.

T-infusion of the mobile phase (isocratic composition) at 500 μL min^{-1} and an infusion flow rate of 10 μL min^{-1} of a 200 ng mL^{-1} mixture of ODV and TRA was analyzed by setting the SV of the DMS cell to 3750 V and scanning a CV window from 20.0 V to 5.0 V. The centroided CV value for each isomer was used in the MRM table for selective monitoring of the most intense MRM transition; *m/z* 264 → 58 (see Table S7).

Determining signal-to-noise ratios for chiral LC×DMS-MS^2:

After demonstrating separation with the chiral LC×DMS-MS^2 method, it was observed that the signal intensity during analysis for a series of wastewater influent extracts was sufficient for quantitation using SPE pre-concentration and internal standards of each isomer. Signals recorded were compared to internal standard responses in the calibration curve and wastewater extracts to generate calibration curves for ODV and TRA, allowing the quantitation of the enantiomers of each drug using the novel chiral LC×DMS-MS^2 workflow. The standards used for this preparation were racemic mixtures of each chiral drug and the respective internal standard. The signal-to-noise (S/N) ratios from this experiment are presented in Table S8, demonstrating sufficient signal being generated at trace analysis concentrations for quantitation, and the potential for LOD/LOQ to be determined for standards with lower concentrations.

S/N ratios were determined using the SCIEX "S-to-N-Script" script built-in to the Analyst software. The script operates by first determining the maximum peak intensity of a specified noise region (S_{noise}) and the maximum peak intensity of the peak of interest (S_{peak}), followed by dividing S_{peak} by the signal intensity of the noise. This is expressed in Eqn S1:

$$\frac{S}{N} = \frac{S_{peak}}{S_{noise}} \qquad \text{(S1)}$$

A noise window of 1 minute prior to elution of the first eluting enantiomer was used, and the same window was selected for each enantiomer for consistency in S_{noise}. In Table S8, the S/N ratios for each enantiomer of ODV and TRA are presented. Note that the S/N ratios of each enantiomer were approximately equivalent for each isomer because the standards used in the calibration curves were racemic mixtures.

Using the LC×DMS-MS^2 set-up, the limit of quantitation (LOQ) for each enantiomer of ODV was determined to be approximately 5.0 ng mL^{-1} as the S/N ratios for each enantiomer is approximately 10. For TRA, the S/N ratios observed for each calibration point was above the threshold for LOQ. The estimated LOQ for each enantiomer of TRA is 0.67 ng mL^{-1} if the S/N ratios from each enantiomer's first calibration point is extrapolated. The greater S/N ratios and superior LOQ observed for TRA relative to ODV can be attributed to a combination of factors, including a twofold difference in intensities of the 264 → 58 transition at equal concentrations (Figure 2; Figure S6), unequal chromatographic peak widths, and variability in noise across CV channels.

Determining resolution of ionogram features in DMS:

To determine the spatial resolution achieved using the DMS, equation S2 was used to relate ionogram features and the width of said features to resolution.

$$Resolution = \frac{2(CV_2 - CV_1)}{FWHM_1 + FWHM_2} \tag{S2}$$

where CV_1 and CV_2 are the centroid CV (in V) required for transmission for each isomer, and the $FWHM_1$ and $FWHM_2$ are the corresponding full width at half-maximum intensity for each isomer. FWHM values were obtained using OriginPro peak fitting algorithm and resolution was then manually calculated.

The results of experiments involving resolution calculations are presented in Table 1.

Table S1: LC method descriptions. All methods held column compartment at 30 °C with a 5 µL injection volume.

Method	Description	Column Specifications	Mobile phase	Gradient
Method A	Reverse-phase for EPI validation	Agilent ZORBAX Extend-C18, 2.1×50 mm, 1.8 µm	MPA: H_2O + 0.1% formic acid MPB: ACN + 0.1% formic acid	0.00 min: 10% MPB 0.10 min: 18% MPB 7.00 min: 100% MPB 10.00 min: 100% MPB 10.10 min: 10% MPB 17.10 min: 10% MPB F = 400 µL min^{-1}
Method B	Chiral LC separation for	Agilent Poroshell 120 Chiral-V	MPA: 1 mM ammonium	Isocratic: 90% MPB for 15 minutes.

	quantitation of enantiomeric	4.6×100 mm, 2.7 µm + Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 mm, 1.8 µm UHPLC Guard Column	acetate in water + 0.6% acetic acid MPB: 100% ethanol	F = 500 µL min^{-1}
Method C	Reverse-phase for quantitation of racemic concentrations	Waters XSelect CSH C18, 2.1×75 mm, 2.5 µm	MPA: H_2O + 0.1% formic acid MPB: ACN + 0.1% formic acid	0.00 min: 0% MPB 0.10 min: 0% MPB 7.00 min: 70% MPB 7.10 min: 100% MPB 13.00 min: 100% MPB 13.10 min: 0% MPB 23.00 min: 0% MPB F = 300 µL min^{-1}

Where MPA represent mobile phase A, MPB represents mobile phase B, and F represents the flow rate.

Table S2: MS Conditions for chiral LCxDMS-MS/MS workflow. Conditions are similar for infusion experiments.

Source Parameter	Source Setting Input	DMS/MS^2 Parameters	DMS Setting Input
CUR	20 psi	DT	150 ˚C (423K)
GS1	30 psi	CAD	High = 12 psi
GS2	80 psi	MDC	1.5%
TEM	300 °C (573 K)	EP	10 V
IS	5500 V	CXP	15 V

Table S3: Collision energies monitored during the LC-MS-EPI experiment and the associated fragments with an optimal collision energy approximately at the corresponding collision energy.

CE / V	Fragment m/z to be monitored

20	246, 201
35	159, 145, 133, 121, 58
45	131, 121, 107, 105, 81
65	127, 103, 91, 81, 79
85	115, 79, 77
110	89, 51

Table S4: and computed collision cross-sections (CCS) for ODV and TRA. Computed CCS's were obtained using Boltzmann-weighted CCS outputs from MobCal-MPI 2.0. The workflow used to obtain the computed CCS is presented elsewhere.[1] Error associated with experimental and computed CCS is represented by a single standard deviation.

Method of obtaining CCS	ODV CCS / Å^2	TRA CCS / Å^2	Absolute % difference
Reference[2]	166.2	161.1	3.4
Experimental	166.0 ± 0.5	160.9 ± 0.3	3.1
Computed	163.8 ± 1.3	158.0 ± 0.7	3.6

Table S5: Binding energies of subsequent additions of isopropyl alcohol (IPA) for ODV and TRA. The lowest energy conformations of each gas-phase cluster was used as the representative for the binding energy.

# of IPA in solvent cluster	ODV + # of IPA binding energy / kJ mol^{-1}	TRA + # of IPA binding energy / kJ mol^{-1}

1	14.7	16.1
2	18.5	6.5

Table S6: Multiple reaction monitoring (MRM) table for LC-MS/MS analysis of ODV + TRA. Entrance potential (EP) was set to 10 V.

Q1 mass / Da	Q3 mass / Da	Dwell time / ms	ID	DP / V	CE / V
264	246	10	TRA-01	70	16
264	58	10	TRA-02	70	45
264	246	10	ODV-01	80	18
264	58	10	ODV-02	80	50
268	58	10	TRA13C-D3	80	59
270	107	10	ODV-D6	70	47

Table S7: Multiple reaction monitoring (MRM) table for LC×DMS-MS/MS analysis of ODV + TRA at a separation voltage of 3750 V, DMS cell temperature of 150 °C and 1.5% IPA modifier. Compensation voltage (CV) values for transmission were chosen using Figure 3 in the main body. Entrance potential (EP) was set to 10 V.

Q1 mass / Da	Q3 mass / Da	Dwell time / ms	ID	DP / V	CE / V	CV / V
264	246	10	TRA-01	70	16	-2
264	58	10	TRA-02	70	45	-2
264	246	10	ODV-01	80	18	-7
264	58	10	ODV-02	80	50	-7
268	58	10	TRA13C-D3	80	59	-2

270	107	10	ODV-D6	70	47	-7

Table S8: Tabulated average signal-to-noise ratios (in duplicate) for each enantiomer in low concentration standards used for calibration curve generation.

Concentration / ng mL^{-1}	Signal-to-noise ratio (S/N) (S,S)-TRA	 (R,R)-TRA	 (S)-ODV	 (R)-ODV
0.0	0	0	0	0
1.0	15	16	4	3
5.0	22	24	10	10
10.0	53	53	24	21

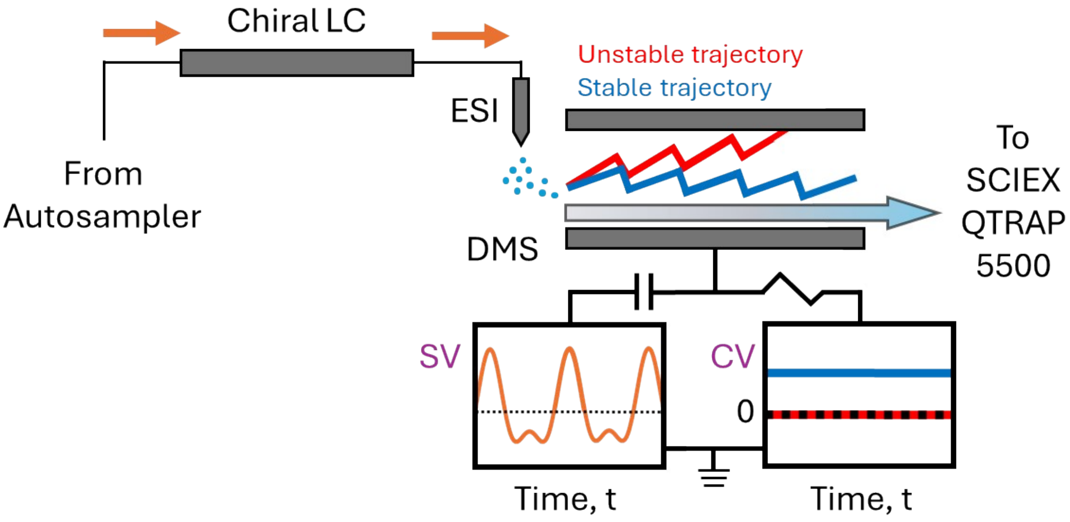


Figure S1: Experimental set-up schematic showing the sample path before MS analysis. In the DMS schematic, the red zigzag trace represents unstable ion trajectories and the blue zigzag trace, stable trajectories following application of the correcting CV at the specific SV.

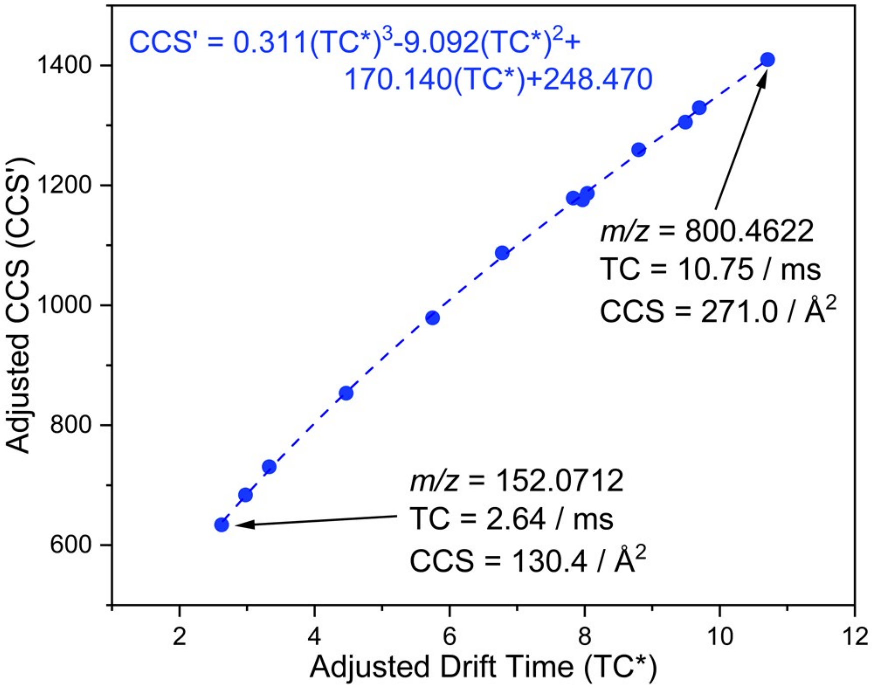


Figure S2: Sample calibration curve of Waters Major Mix delivered via direct infusion in positive ion mode for TWIMS using wave height (WH) = 34 V and wave velocity (WV) = 700 m/s. The plot demonstrates the bounds of the calibration range for the parameters used.

The adjusted drift time is defined by:

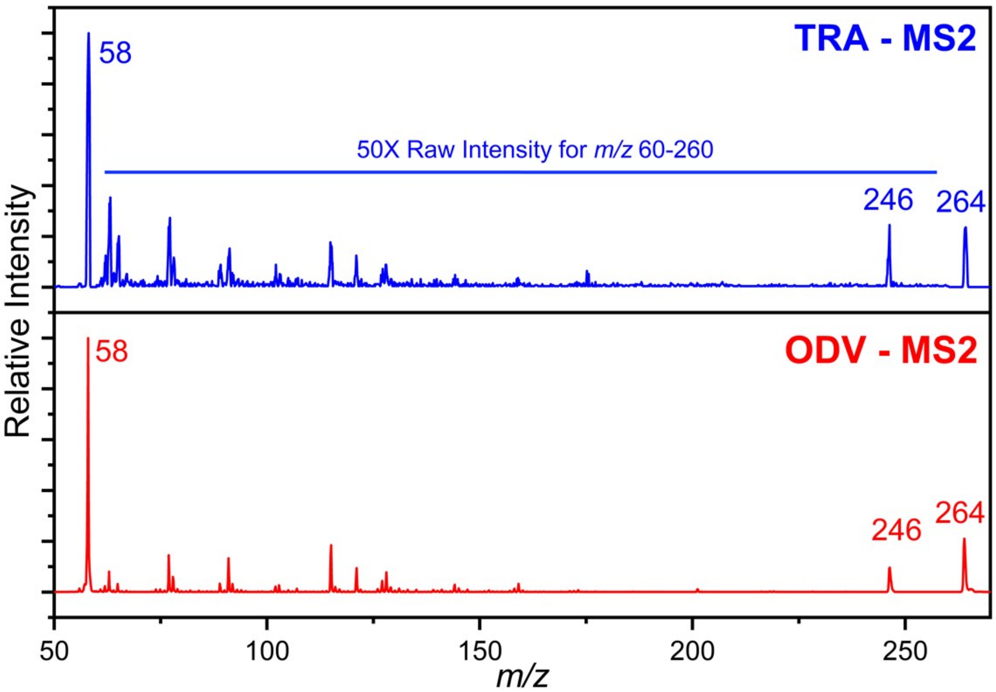
$$TC^* = TC - \frac{edc * sqrt\left(\frac{m}{z}\right)}{1000}$$

where *TC* is the raw drift time, *edc* is the duty cycle of the instrument, and *m/z* is the mass-to-charge of the ion of interest.

The adjusted CCS is defined by:

$$CCS' = \frac{CCS * sqrt(Reduced\ mass)}{charge}$$

where *CCS* is the native collision cross-section of the ion of interest and the square root of the reduced mass represents the inertial mass of the bath gas and ion interaction. Both of these corrections are commonly applied to TWIMS measurements.[3]



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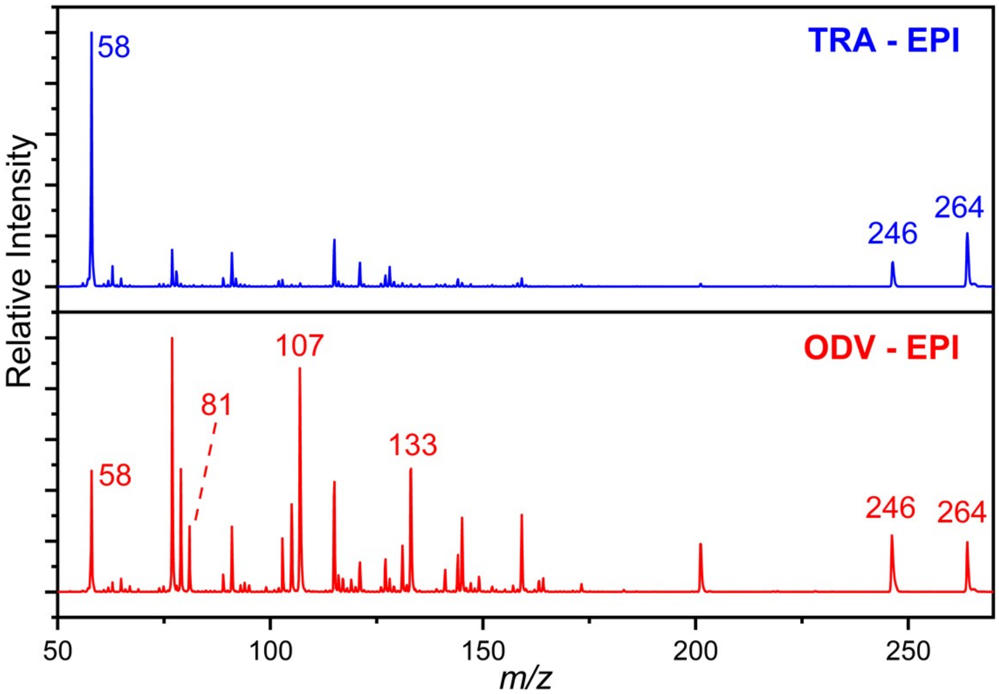

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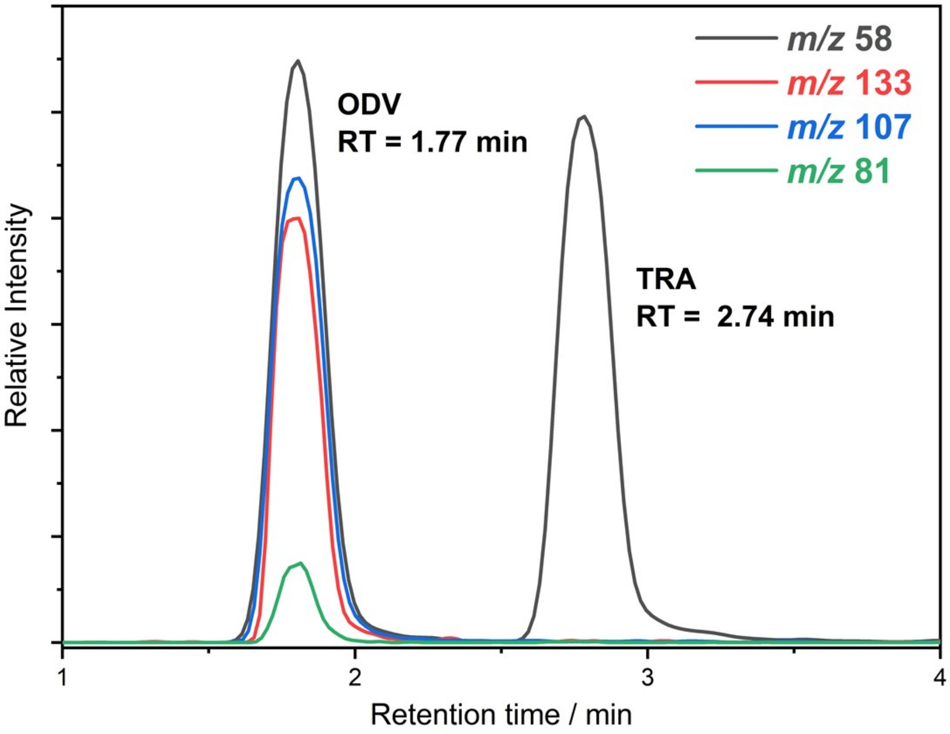

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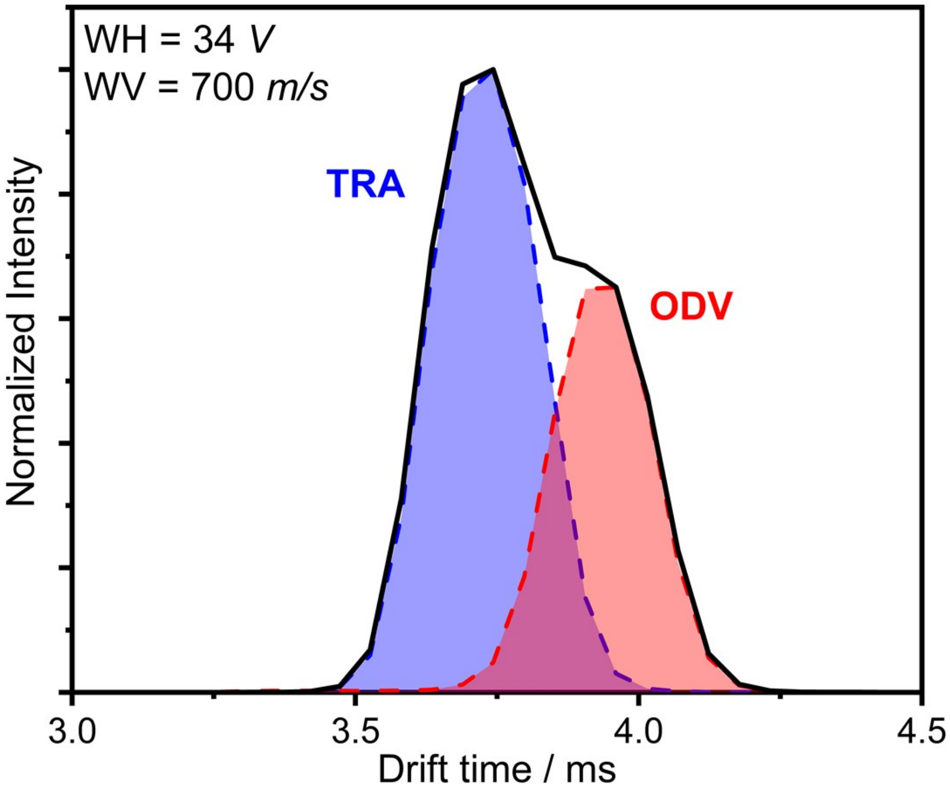

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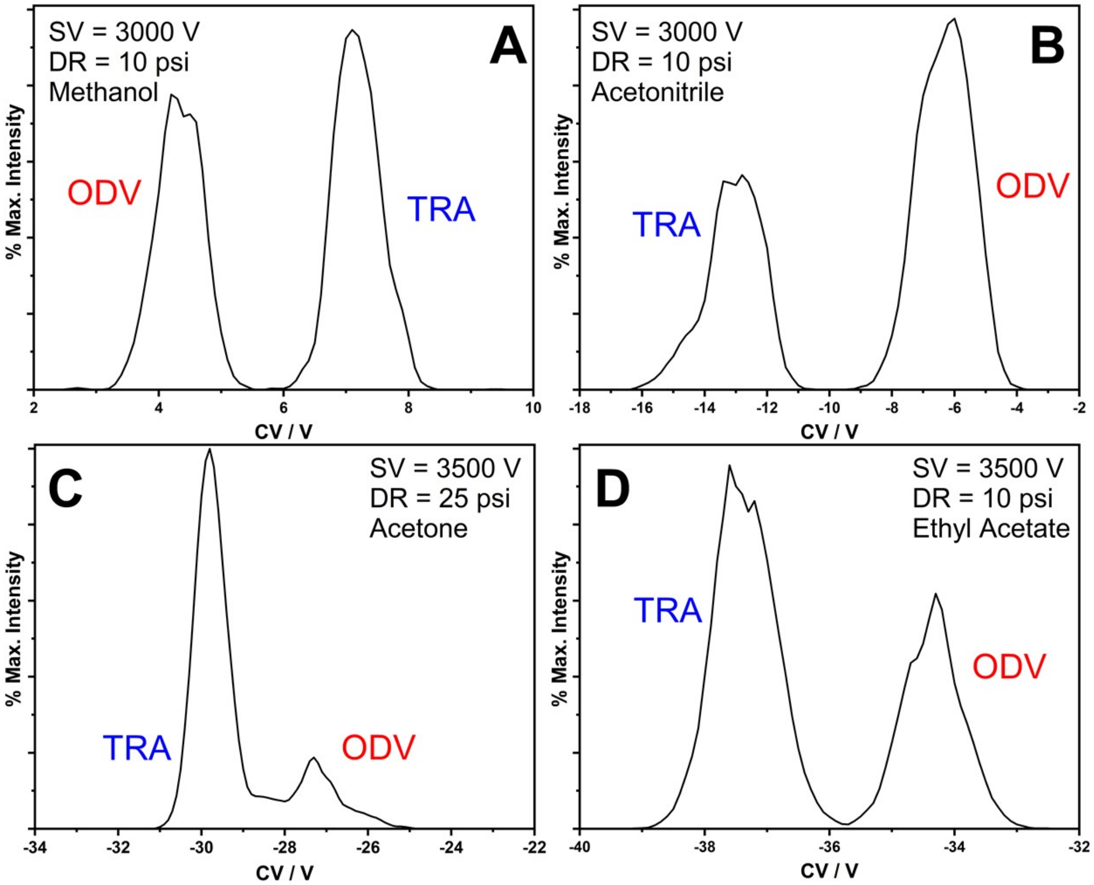

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(1) Haack, A.; Ieritano, C.; Hopkins, W. S. MobCal-MPI 2.0: An Accurate and Parallelized Package for Calculating Field-Dependent Collision Cross Sections and Ion Mobilities. *Analyst* **2023**, *148*, 3257–3273. https://doi.org/10.1039/d3an00545c.

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