Quantitation of Isomers by Multi-CV FAIMS-MS Scans

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ABSTRACT

Purpose: Development and evaluation of a method for quantitation of isomers or, more generally, isobaric LC-MS interferences, by FAIMS-MS when baseline separation of the interfering compounds by the FAIMS apparatus is not possible in practice.

Methods: Leucine and isoleucine are used as representative test compounds for the method. After acquiring FAIMS CV tuning curves for pure standards, mixture samples are analyzed by performing MS scans (SIM or SRM) at multiple CV setpoints determined in advance by inspection of the tuning curves, iterating through the CVs repeatedly. The contributions of the individual isomers or interferences are then inferred from the multi-CV data by a procedure formally equivalent to multilinear regression.

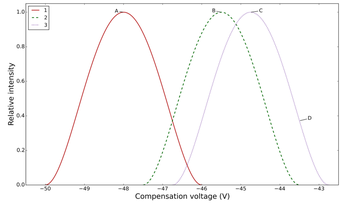
Results: The concentration of leucine in a leucine-isoleucine mixture was estimated with accuracy within 10% of the total concentration of both isomers across the range of mixture composition (from 100% isoleucine to 100% leucine) using a basic least-squares method of inference. Isoleucine concentrations were systematically undermeasured by 10–20%, possibly due to an overestimated scale factor, showing a need for further development of calibration procedures for this method.

INTRODUCTION

As a separation technique compatible with and orthogonal to liquid chromatography and mass spectrometry, FAIMS (high-field asymmetric ion mobility spectrometry, sometimes also called DMS) is useful in separating compounds that cannot be distinguished or difficult to distinguish or separately quantitate by mass spectrometry alone. An important and long-established but underutilized application of this is to structural isomers[1-3]. By different mechanisms dependent on the class of molecule, structural isomers difficult to quantitate in mixtures by analysis of MS/MS peak ratios can have different differential mobility allowing their separation by application of selected compensation voltages.

In practice, full baseline separation cannot be attained (as it was in Ref. 1) by increasing the dispersion voltage (DV) until compensation voltage (CV) tuning curve peaks separate; separation is strongly dependent on carrier gas composition[2-4], electrode temperature, and electrode geometry. It may not be possible to find universal conditions that perform the desired separations for large libraries of (for example) metabolites, pesticides, or other analytes. The problem, as we see below, grows with the number of isomers of interest.

Figure 1. Constructed CV spectra for a representative scenario. Three compounds of interest separate by FAIMS but not fully. One (#1, solid, red) is separate from the others at its optimum (A). Another (#3, dotted, indigo) separates fully only when its signal is attenuated by 66% (D). The third (#2) can never be fully separated from the others under these conditions.



If only two of the three compounds were present in the scenario represented by Figure 1, separation could be achieved by selecting one CV for each compound. For #1 and #3 the problem is simple as they are separate from each other at their respective optima A and C. For #2 and #3 to perform the same analysis would require a 66% attenuation of the signal, selecting point D and its counterpart on curve 2. For a mixture of all three compounds, points A and D could be selected to quantitate #1 and #3, but there is no CV for compound #2 where one or the other do not.

Here we describe a method for acquisition and analysis which relaxes the need for full separation of isomers by FAIMS. FAIMS spectra are obtained for pure standards, then MS scans of the same mass are done over a set of M CVs at which the transmission of the various N isomers (M≥N) through the FAIMS device differs. We then use a mathematical procedure formally equivalent to multilinear regression, to use all of the available information to infer the contribution of each isomer to the ion beam.

MATERIALS AND METHODS

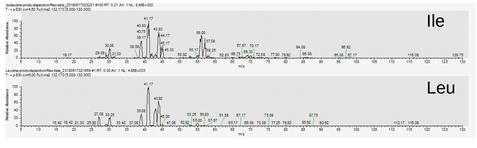
Sample Preparation

1 mg/mL stocks of *l*-leucine and *l*-isoleucine in 50:50 water:methanol (0.1% formic acid) were prepared from reagent-grade powders obtained from Sigma-Aldrich. These were in turn used to prepare 10 µg/mL pure and mixed samples for analysis.

Instrumentation and Data Acquisition

Measurements were performed using a pre-release Thermo Scientific™ FAIMS prototype interface apparatus and a Thermo Scientific™ TSQ Altis™ mass spectrometer, running the samples in positive ion polarity and using dry nitrogen as the carrier gas. CV tuning curves were acquired using the CV Scan Tool feature of version 3.1 of the instrument control software. Multi-CV SIM scan data were acquired using a custom instrument control script.

Figure 2. Product ion spectra for isoleucine (top) and leucine (bottom). 40 eV collision energy, collision cell pressure 2.0 mTorr.



Samples were delivered to the mass spectrometer's HESI source by continuous infusion using a syringe pump. Between samples, the infusion line and ionization source were rinsed with 50–100 µL of methanol followed by a minimum of 100 µL of the new sample solution. Adequacy of this rinsing was confirmed during design of the experiment by inspection of product ion spectra (Figure 2). So that excess methanol or water vapor present due to the rinse procedure would not serve as a dopant gas, absolute intensity of the SIM signal at each of the selected CVs was monitored and allowed to settle before beginning data acquisition. During the design of the experiment, the supplemental FAIMS gas flow was determined by collecting CV tuning curves for the pure standards at multiple setpoints (not shown), noting the setpoint at which the curve shapes and apices would stop shifting and choosing a higher flow rate than this to provide some margin.

Experimental conditions were as follows:

Ion Source	
Spray voltage	3500 V
Sheath gas	4 (arb)
Aux gas	0 (arb)
Vaporizer temperature	50 °C
Flow rate	500 nL/min

FAIMS	
DV	–5000 V
Inner electrode temperature	70 °C
Outer electrode temperature	100 °C
Supplemental gas	2.5 L/min

Mass Spectrometer	
Capillary temperature	300 °C
Mode	Q1 SIM
Mass	132.17 Da
Dwell time	100 ms

Selected CVs

From the FAIMS CV spectra presented in Figure 4, five CVs were chosen for the scan:

A. Outer 50% level for isoleucine: –0.44 V
B. Optimum for isoleucine: 1.2 V
C. Crossing: 1.58 V
D. Optimum for leucine: 1.9 V
E. Outer 50% level for leucine: 3.6 V

Data Analysis

SIM intensity records were exported using custom instrument control scripts for offline analysis in an interactive Python session using the methods detailed in the next section.

DATA ANALYSYS

Motivation

Considering the scenario in Figure 1, we recall that we can quantitate isomers 1 and 3 without influence of the others. If we take an additional measurement at point C, which we will call Φ_C (ion flux at C, we can obtain the contribution of isomer 2 by subtracting out the flux of isomer 3. If we call the height of the normalized CV tuning curve for compound 3 at point C $w_{C,3}$(w for "weight"), the total signal for isomer 2 is

$$\phi_2 = \frac{\Phi_C - w_{C,1}\phi_3}{w_{C,2}}$$

If we substitute so ϕ_3 is represented in terms of the measured ion flux Φ_D, we obtain

$$\phi_2 = \frac{\Phi_C - w_{C,1}\frac{\Phi_D}{w_{D,3}}}{w_{C,2}}$$

If isomer 3 is in great excess relative to isomer 2, a point to the left of C might provide better discrimination, but the contribution of isomer 1 would also have to be subtracted in a self-consistent way. Moreover, it is difficult to determine in advance (relative abundances unknown) what set of N CVs provides the best balance between sensitivity and discrimination for N compounds. It is better to collect information on a larger set than is necessary, but if all of it is included the problem becomes overdetermined.

Rather than piece together estimates term-by-term as above, we can take a more general approach, as follows:

Inference of Isomer Intensities from Measured Ion Fluxes

If N isomers are potentially present in the mixture and measurements are taken at M CVs, then the total intensity at each CV setting is given by the linear system

$$\begin{aligned}\Phi_1 &= w_{1,1}\phi_1 + w_{1,2}\phi_2 + \cdots + w_{1,N}\phi_N \\ \Phi_2 &= w_{2,1}\phi_1 + w_{2,2}\phi_2 + \cdots + w_{2,N}\phi_N \\ &\vdots \\ \Phi_M &= w_{M,1}\phi_1 + w_{M,2}\phi_2 + \cdots + w_{M,N}\phi_N\end{aligned}$$

Calling the weight matrix **w**, the vector of measured intensities **Φ**, and the vector of unknown intensities of the individual isomers **ϕ**, this is noted more simply as the matrix equation

$$\mathbf{\Phi} = \mathbf{w}\boldsymbol{\phi}$$

If **w** is square then solution is elementary provided that none of the CV tuning curves are identical, in which case the experiment needs to be modified to introduce some separation between all isomers. If (as recommended) M≥N and the problem is well-conditioned (no identical tuning curves), the problem is overdetermined but a number of approaches are available to find a statistically good solution. A least-squares solution can be obtained using the Morse-Penrose pseudoinverse

$$\boldsymbol{\phi} = \mathbf{w}^{+}\boldsymbol{\phi} = (\mathbf{w}^{\mathrm{T}}\mathbf{w})^{-1}\mathbf{w}^{\mathrm{T}}$$

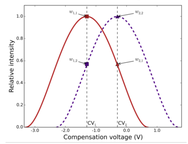
Although we did not cast the problem as a multilinear regression, it should be noted that this is identical to the formula for multilinear regression. This approach does not account for the variation of measurement noise with signal intensity, nor for the nonnormal distribution of the measurement noise. GLM algorithms can provide estimates accounting for these properties, with considerably more computational cost and conceptual complexity including the need determine a link function relating the noise distribution to the signal intensity. This is beyond the scope of this poster; the results presented are inferred using the least-squares approach given above.

Worked Example

Figure 3, below, illustrates how the weight matrix is obtained from a set of CV tuning curves in a simple 2×2 case:

Figure 3. Example of how to obtain a weight matrix from CV tuning curves in the simple case where there are two isomers in the mixture and the measurement will be taken at two CVs.

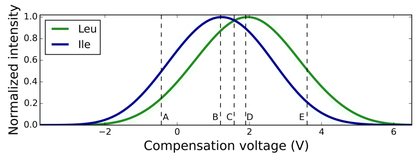
Here $\mathbf{w} = \begin{pmatrix} w_{1,1} & w_{1,2} \\ w_{2,1} & w_{2,2} \end{pmatrix}$



RESULTS

CV Tuning for Leucine and Isoleucine

Figure 4. CV tuning curves for leucine and isoleucine measured under our experimental conditions. Curves were constructed by averaging five of the smoothed tuning curves output by the TSQ Altis™ Tune™ CV scan tool. CVs selected for the analysis have been marked with vertical lines.



Under our conditions, there is a clear difference between the transmission of Leu+H⁺ and Ile+H⁺ through the FAIMS apparatus but true separation cannot be attained without attenuating more than 95% of the beam by detuning the CV away from its optimum. The tuning curves are separated by 0.7 V, with FWHM 3.4 V.

Transmission Characteristics of Mixtures

Figure 5 below shows the raw signal at the various CV settings for different proportions between leucine and isoleucine. The consistent upward trend is most likely due to more efficient ionization of leucine under our conditions. Since the weight matrix is constructed with reference to the tuning curve peak, this is calibrated out by scaling the inferred ion flux for an isomer by the response of a pure sample of known concentration at its CV optimum.

Figure 5. Raw intensity traces for 10 µg/mL leucine-isoleucine mixtures at selected CVs. Top: full range. Bottom: rescaled to show change in the CVs most informative of relative concentration.

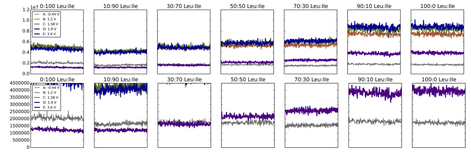
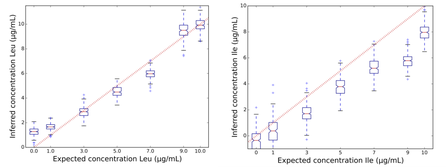


Figure 6. Concentration of leucine and isoleucine in solution, inferred from the multi-CV scan data shown in Figure 5 using the least-squares method. Statistics represented by the boxes and whiskers are based on a minimum of 200 scans per sample.



CONCLUSIONS

The accuracy of estimates of leucine concentrations presented in Figure 6, to within 10% of the total concentration and often much better, shows that this method of isomer quantitation is reasonable and not completely overwhelmed by noise.

Systematic underestimation of isoleucine concentrations is of concern. That the estimates appear to follow an alternative trend line, together with the deviation of the trend for leucine upwards from the expected value as its fraction of the mixture approached zero points to miscalibration as the probable reason. Calibration and QC procedures need to be studied and established. We note also that oversmoothing of the CV tuning curves, depressing their values at the apex, results in an erroneous normalization and propagates through the calculation due to its effect on the weight matrix. Whether choosing a narrower kernel bandwidth or different smoothing method or excluding CVs near the apex from the measurement reduce the observed systemic errors should be investigated.

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TRADEMARKS/LICENSING

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