Cerno Application Note

Extending the Limits of Mass Spectrometry

Direct Quantitation of Complex Mixtures of Isotope Labeled Compounds

Introduction

Isotope labeled compounds and associated experiments play a key role in the investigation of absorption, distribution, metabolism and excretion (ADME) properties of new chemical entities as well as metabolic flux or quantitative bioanalytical analysis. The labeled compounds typically contain some amount of native compounds, the relative amount of which can introduce systematic errors in subsequent analysis and reported results. When measured with MS, there is severe MS signal overlap between the labeled and un-labeled species, posing a quantitative challenge even for high resolution MS. In this note, we will demonstrate the use of a highly effective direct MS analysis approach to this problem through mass Spectral Accuracy[1] analysis.

Experimental

Two commercially available compounds (Diclofenac), one with stable ^{2}H labeling and one with multiple (uniform) ^{14}C labeling, were used to evaluate the method. First, various pure calibration standards were acquired under normal operating conditions on a Waters Xevo TQ-S quadrupole LC/MS and under varying resolving powers on a Thermo Fisher Scientific LTQ/Orbitrap to determine the optimal Spectra Accuracy achievable. All data was processed offline using MassWorks™ software (Cerno Bioscience). The Spectral Accuracy of a known standard is an excellent measurement for how well the MS system is operating in terms of linearity and spectral integrity, very important for accurate quantitative measurements. Synthetic mixtures of the labeled and unlabeled compounds were then acquired and calibrated with this MS calibration. With the Spectral Accuracy achieved through this calibration, the mutually overlapping MS signals from various labeled and un-labeled ion species in a mixture can be mathematically resolved through least squares regression, with accurate quantitative results for the relative concentration of each ion.

Results and Discussion

Quantitation of labeled isotopes by MS is very challenging due to the fact they are extremely difficult to separate chromatographically and the MS peaks of the A and A+1, …, A+n peaks of the various isotopes overlap such that even high resolution mass spectrometer cannot resolve them. In spite of the various spectral overlap correction schemes proposed in the literature, substantial inaccuracies of as much as ±10% or more have been observed even at high concentrations of 50% (50:50) labeled to un-labeled mixtures, making reliable quantitation of 5-10% mixtures virtually impossible.[2,3] By performing the Cerno TrueCal™ calibration available in MassWorks on the measured MS data, the MS lineshapes are calibrated to a known analytical lineshape which provides for high Spectral Accuracy. This allows for the direct fitting of the calculated "True" mass spectrum of each isotope to the calibrated mass spectrum using a multivariate least squares best fit. The fitting residual also provides a critical diagnostic metric for evaluating the performance of the MS hardware and hence confidence in

cerno BIOSCIENCE

40 Richards Avenue, 3rd Fl. •Norwalk, CT 06854, USA • Tel 203-312-1150
Email: info@cernobioscience.com • Web: www.cernobioscience.com

the results. For convenience, we will refer this method as the True Fit with MultipleXing (multiple spectral components) or simply, TrueFit MX™.

Initial measurements were made using a Thermo LTQ/Orbitrap. It has been shown that to obtain the best Spectral Accuracy, and hence the correct isotopic profile, on this class of instruments, there is an optimal resolution[4] usually around 7,500. For some initial test compounds which contained only C/H/N/O/F, the spectral accuracy values were found to be excellent at above 99.0%. However, with chlorine containing compounds, the spectral accuracy decreased significantly to ~97%. Further experimentation saw an even greater degradation in Spectral Accuracy to 92% with a 50:50 mixture of native and [2H] labeled compounds, corresponding to an 8% spectral error. It is believed that the problem arises mainly from space charge effects at A+2 isotope clusters due to the ion-ion interactions between the high natural abundance of [37Cl] (A+2) and the monoisotope (A) of the double-[2H] labeled ion. When the double-[2H] labeled compound is added in, the total ion abundance surrounding A+2 isotope clusters becomes too high, leading to poor Spectral Accuracy. Naturally, these errors propagated through the analysis and led to quantitation errors of up to ~±10%.

For comparison purposes, it was decided to use a quadrupole instrument to perform the same measurements. While quadrupoles run at much lower resolution, they are known to be free of these space charge errors plaguing the ion trap type of MS instrumentation. TrueFit MX does not require fully resolved isotope bands and it was felt this would be the most likely approach to succeed. The radiolabeled test compound, Diclofenac, shown in Figure 1, had an specific activity of 62.7 mCi/mmol according to the specification sheet from the supplier (PerkinElmer). The specific activity measures the overall [14C] radioactivity, likely contributed by variously labeled species containing between 0 and 6 [14C] around the aromatic ring. A series of synthetic test mixtures containing different ratios of the native and the purchased labeled Diclofenac was volumetrically prepared as shown in Table 1 below.

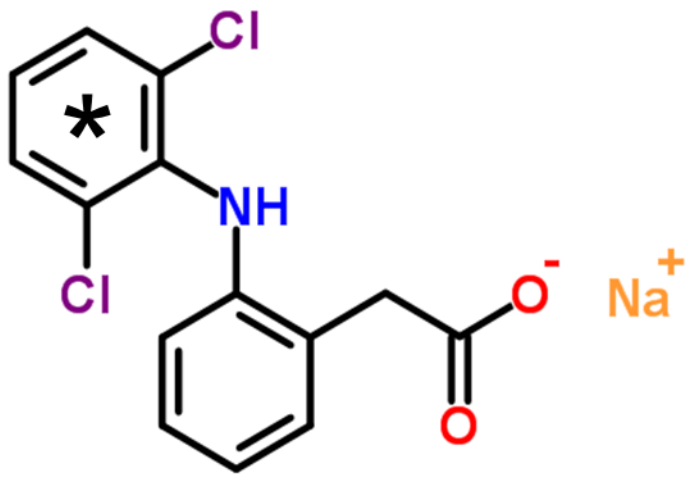

Figure 1. Diclofenac Sodium chemical structure. The aromatic ring labeled "" contains [14C] in a mixture where the ring can contain between 0 and 6 [14C], i.e., a mixture of seven possible species with 0-6 [14C] labels.*

Table 1. Synthetic mixture series measured by LC/MS where A_0 is the native Diclofenac and A_L is the radio-labeled Diclofenac.

Mix #	A_0	A_L
1	1.00%	99.00%
2	2.00%	98.00%
3	4.00%	96.00%
4	8.00%	92.00%
5	12.00%	88.00%
6	20.00%	80.00%
7	30.00%	70.00%
8	50.00%	50.00%

Figure 2 below shows a screen shot of the LC/MS run and the CLIPS formula search parameters set up to perform the mixture analysis through TrueFit MX. The search is set to perform a formula search for the native Diclofenac which includes all the elements for that compound. In the search dialog, an "Ion Series" is also entered which specifies the number of [14C] substitutions from 1 through 6 (as indicated by -C +[14C]). This indicates to the software to perform the multivariate best fit of all 7 species including the native form.

Figure 3 below shows the overlay of the calibrated mass spectrum and the calculated "True" mass spectrum from the multivariate fit of all 7 species for the 100% unlabeled (cold) and the 100% hot Diclofenac. In both cases, the Spectral Accuracy is excellent, on the order of 99%. The cold Diclofenac is calculated to be indeed 99.4% cold while the Hot is calculated to give a specific activity of 62.8% based on the resulting relative concentrations for all [14C]-labeled species which is in excellent agreement with the 62.7 mCi/mmol value provided by the supplier.

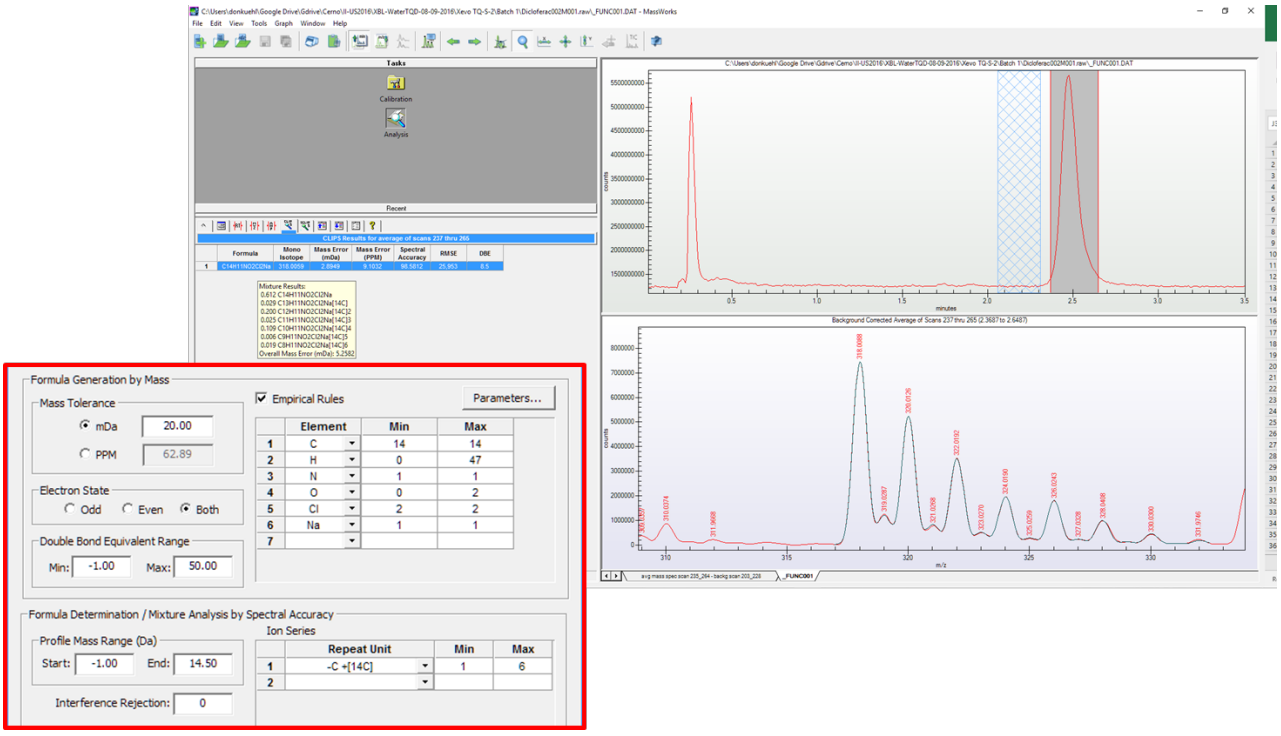

Figure 2. The main screen graphics shows the chromatogram (upper) and the mass spectrum (lower) of Diclofenac. The CLIPS search parameters are set to provide the best fit using all 7 possible species.

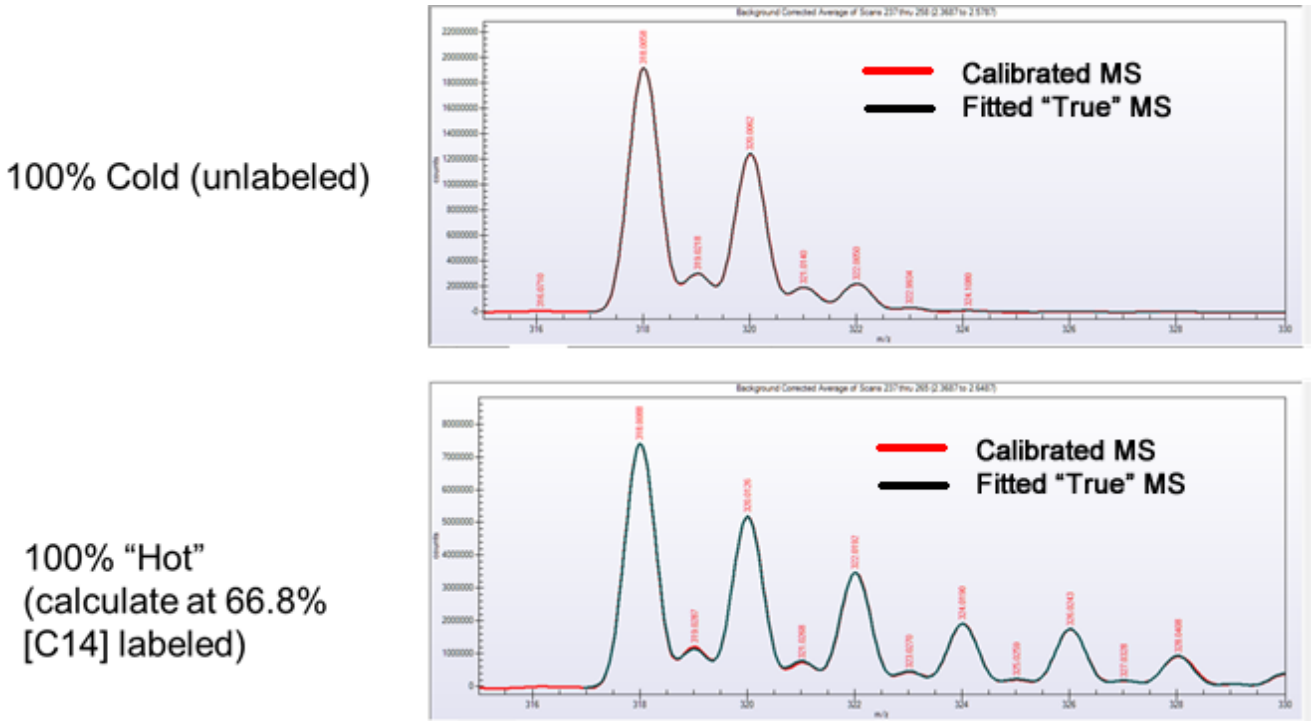

Figure 3. Top graph shows the calibrated and fitted spectra of 100% cold Diclofenac. The bottom graph shows the fit for the 100% hot.

To better visualize the TrueFit MX fitting, Figure 4 shows an overlay of all species and their respective spectral contributions to the fit. As part of this fitting process, the relative quantity of each species is also calculated. It should be noted that it is very difficult to perform this analysis with any other analytical technique except perhaps NMR, which works with only a limited set of label types, requires large sample volumes and higher concentration, and can be very time consuming.

Figure 5 shows a number of quantitative bar charts and statistical plots designed to illustrate the performance of the TrueFit MX method. It should be emphasized that the standard error across all concentrations does not exceed 1.76% even down to 1% (1:99 mixture) concentration. This should be contrasted against current methods where errors are as large as 10% at the 50% (50:50 mixture) level and are thus incapable of producing reasonable results below the 10% (1:9 mixture) level[2,3].

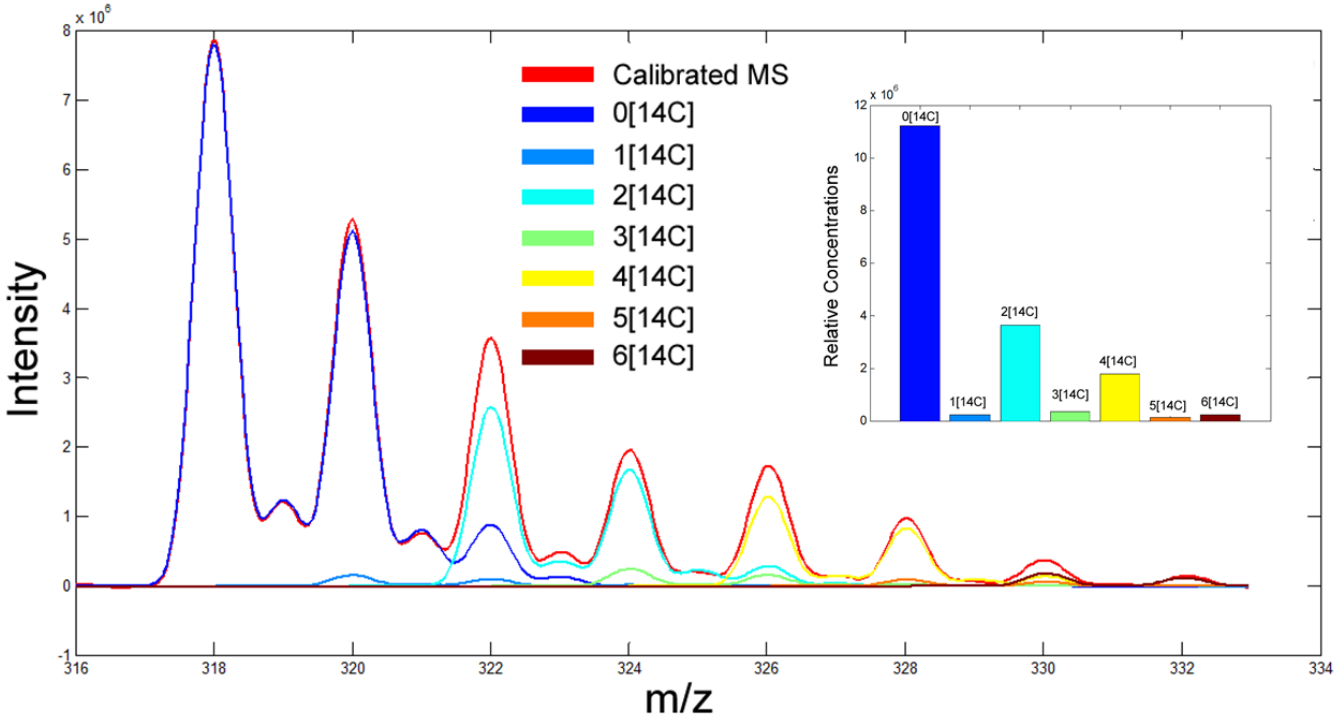

Figure 4. An overlay of the calibrated mass spec of hot diclofenac and the series of ion species fitted to the spectrum.

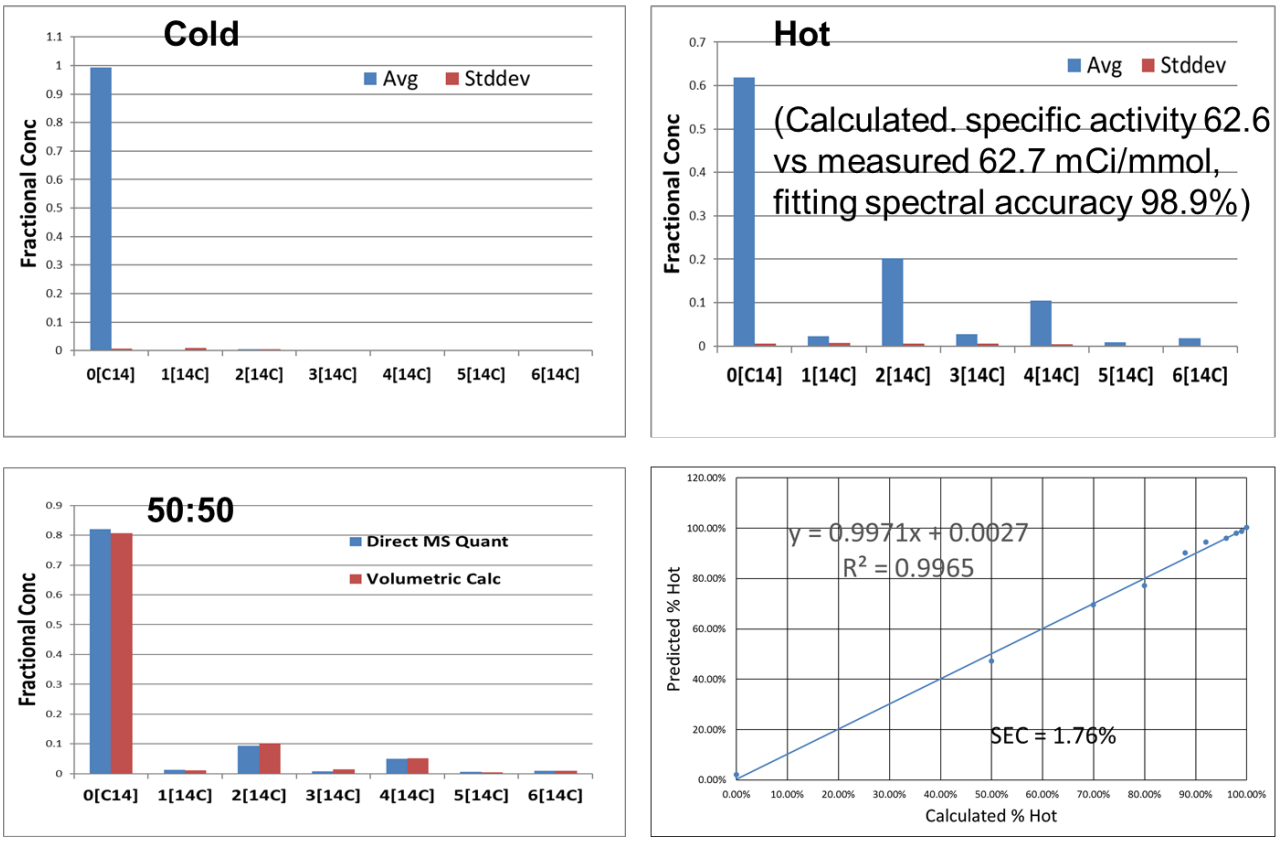

Figure 5. Quantitative results for the cold Diclofenac (upper left) show excellent statistics in agreement with actual value of 100%. The 100% hot (upper right) quantitates all the labeled and unlabeled species which when converted agrees very well with the measured specific activity value. The 50:50 mixture of Hot:Cold (lower left) compares the direct MS quantitation results with the volumetric values. The Predicted and calculated % cold show a good linear correlation (lower right) with an R^2 of 0.9965 and a standard error of calibration (SEC) of 1.76%.

Conclusion

A new method for the MS quantitation of complex isotope mixtures is shown to be far superior to existing MS methods and provides for accurate quantitation approaching the 1% level. The TrueFit MX method relies on the ability to measure quality MS data with good Spectral Accuracy and care must be taken of instruments that are susceptible to non-linear errors, such as space charge interactions for closely space isotope masses. Furthermore, the method is simple to perform, does not require high resolution instrumentation, and produces fitting metrics (Spectral Accuracy) that directly validate the quality of the results, minimizing the possibility of reporting incorrect quantitative values.

Acknowledgement

Special thanks Jianwei Zhao, Peter L. Wang, Dawei Zhou & Xinping Fang of XenoBiotic Laboratories Inc. (part of WuXi AppTec), Plainsboro, NJ, USA for performing the LC/MS experiments.

References

[1] Y. Wang, M. Gu, "The Concept of Spectral Accuracy for Mass Spectrometry", Analytical Chemistry, Sept 1, 2010, Vol. 82, No. 17.

[2] Charles S. Elmore, David J. Schenk, Robert Arent and Lee Kingstona, "Evaluation of UV-HPLC and mass spectrometry methods for specific activity determination", J. Label Compd. Radiopharm., 2014, 57 645–651.
[3] David J. Schenk, William J. S. Lockley, Charles S. Elmore, Dave Hesk and Drew Roberts, "Determining the isotopic abundance of a labeled compound by mass spectrometry and how correcting for natural abundance distribution using analogous data from the unlabeled compound leads to a systematic error", J. Label Compd. Radiopharm., 2016, 59 136–146.
[4] Erve, J.C.L., Gu, M., Wang, Y. et al. "Spectral accuracy of molecular ions in an LTQ/Orbitrap mass spectrometer and implications for elemental composition determination", J. Am Soc Mass Spectrom. (2009) 20: 2058.