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# Correction of Precursor and Product Ion Relative Abundances to Standardize CID Spectra and Improve Ecom50 Accuracy for Non-Targeted Metabolomics

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**Correction of Precursor and Product Ion Relative Abundances to  
Standardize CID Spectra and Improve Ecom<sub>50</sub> Accuracy for Non-  
Targeted Metabolomics**

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**B.S., Ramapo College of New Jersey, 2010**

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**APPROVAL PAGE**

**Masters of Science Thesis**

**Correction of Precursor and Product Ion Relative Abundances to Standardize  
CID Spectra and Improve Ecom<sub>50</sub> Accuracy for Non-Targeted Metabolomics**

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## Abstract

Quantitative biases in the abundance of precursor and product ions due to mass discrimination in RF-only ion guides results in inaccurate collision induced dissociation (CID) spectra. We evaluated the effects of collision cell RF voltage and collision energy on CID spectra using ten singly protonated compounds (46 – 854 Da) in an orthogonal acceleration time-of-flight mass spectrometer. The relative ion transfer efficiency, i.e. the relative amount of ions transferred through the ion guide at any particular RF voltage was shown to be dependent on the ion's  $m/z$ . We (Dubey, Hill, Lai, Chen, & Grant, 2014) developed an algorithm to correct for the mass discriminating effects of RF voltage on CID spectra. The algorithm was tested for both precursor and product ions at multiple RF voltages and collision energies in order to ensure reliability. Our results suggest that compounds that generate major product ions with  $m/z$  values <150 have peak intensities that deviate substantially from their actual abundance. This has implications for small molecule metabolomics research, particularly for studies that rely on CID spectra matching methods for structure identification.

## Introduction

The use of mass spectrometry (MS) for metabolomics research has significantly increased over the last 10 years and multiple studies have provided qualitative and quantitative data for metabolites in cells, tissues and whole organisms (Dettmer et al. 2007; Milne et al. 2013; Kaddurah-Daouk et al. 2008). However, despite recent analytical and computational advances, metabolite identification remains a significant challenge that continues to limit the utility of non-targeted (discovery) metabolomics (Hamdalla et al. 2013). Non-targeted metabolomics studies generally use gas or liquid chromatography and tandem MS to identify endogenous compounds (Commisso et al. 2013). This typically involves obtaining accurate mass precursor ion data as well as collision induced dissociation (CID) spectra using a quadrupole or hexapole ion guide collision cell and a time-of-flight (TOF) mass analyzer (Guzowski Jr & Hieftje 2001; Vinayavekhin & Saghatelian 2001). Accurate mass data are then used to search biochemical databases such as HMDB (Wishart et al. 2009), METLIN (Tautenhahn et al. 2012), MassBank (Horai et al. 2010), IDEOM (Creek et al. 2012) and PubChem (Dunn et al. 2013). The experimental CID spectrum of the unknown (often referred to as an MS/MS spectrum) can also be used for searching databases, and many databases (HMDB and METLIN for example) include CID spectra for this purpose.

The CID spectrum of a precursor ion is determined by multiple factors. The internal energy of the precursor ion has perhaps the most profound effect on the CID spectrum. The internal energy of the individual bonds relative to the total internal energy of a molecule determines the extent of fragmentation. Colliding molecules with inert gases is a common method to increase the internal energy of a molecule and induce CID (Vékey 1996). The molecular weight of the collision gas and the collision energy of the precursor ion determine the energy imparted to the



molecule during collision. As energy is conserved between collisions, heavier collision gases produce greater fragmentation as compared to lighter gases at the same pressure and collision energy. The maximum energy that can theoretically be absorbed in a single collision is determined by the center of mass energy ( $E_{com}$ ), which is a proportion of the precursor ion's kinetic energy (collision energy) determined by the ratio of the mass of the collision gas to the total mass of the precursor and collision gas molecules as shown in equation (1).

$$E_{com} = E_k \cdot M_x / (M_x + M_g) \quad (1)$$

Where  $E_k$  is the kinetic energy of the precursor ion,  $M_x$  is its mass in Daltons (Da) and  $M_g$  is the mass of the collision gas.

Collisions convert translation energy into internal energy, which is distributed across the ion to its oscillators. Thus, higher collision energy or a heavier collision gas increases the probability of precursor ion fragmentation as more energy is absorbed during the collision (Hill et al. 2012). In addition, increased collision gas pressure increases the probability of multiple collisions resulting in greater fragmentation of the precursor ion.

Secondarily, increased collision gas pressure and collision energy can improve overall sensitivity since sample ion collisions reduce the ion's axial velocity and focus the ion beam. This process, known as collisional cooling, has been extensively reviewed (Guzowski Jr & Hieftje 2001; Thomson et al. 1995). Beyond a certain threshold (depending on instrumental and experimental parameters), however, higher random-direction kinetic energy of molecules can cause more scattering in the collision cell and results in loss of sensitivity. As a result, the gas pressure and collision energy must be optimized based on the  $m/z$  of the sample ions. In addition, collision cell dimensions affect ion resident time which in turn affect the probability

that an ion that has absorbed sufficient energy will dissociate before it exits the collision cell (Hill et al. 2012). Relative precursor and product ion abundances are therefore related to internal bond energies, the conditions of the experiment, and the physical characteristics of the instrument.

In orthogonal acceleration time-of-flight mass spectrometry (oa-TOFMS), mass discrimination occurs during ion acceleration into the mass analyzer, with ion transfer efficiency being proportional to  $(m)^{1/2}$  (where  $m$  = the mass of the ion) (Guilhaus 1994). Conversely, the approximate electron yield of a micro-channel plate (MCP) detector is inversely proportional to the square root of the mass of the detected ion (Zimmer 1997). Thus, it appears these effects roughly compensate for each other and may not play a significant role in determining relative ion intensities for any particular ion. The overall efficiency ( $F$ ) of an oa-TOFMS mass analyzer is given by equation 2 (Guilhaus 1994).

$$F = T_g Y_d L_{ion} f (2m/zeV_{beam})^{1/2} \quad (2)$$

Where  $T_g$  is the efficiency of the transmission grids,  $Y_d$  is the detection yield of the detector,  $L_{ion}$  is the length of the ion beam sampled,  $f$  is the sampling frequency,  $m$  is the mass of the ion,  $z$  is the charge of the ion,  $e$  is the electron charge and  $V_{beam}$  is the velocity of the beam. Therefore, differences in the efficiency of transmission grids, acceleration voltage or electron yield between ions will likely effect ion peak intensities in CID spectra. Similarly, differences in ion optics between instruments would be expected to result in variations in ion peak intensities.

Hill et al. demonstrated the correlation of  $E_{com50}$  values, i.e. the energy required to fragment 50% of a precursor molecule, between instruments when comparable RF voltages in the collision cells were used (Hill et al. 2012). The  $E_{com50}$  value of a compound depends on its ‘survival

yield' (ratio of precursor ion to sum of product and precursor ions) and as such, is affected by all ion peak intensities in CID spectra. Hill et al found low  $m/z$  precursor ions were most efficiently transferred through the collision cell at lower RF voltages while larger  $m/z$  precursor ions were more efficiently transferred at higher RF voltages. Similar observations were made by Guzowski and Hieftje in a study characterizing RF only hexapole ion guides (Guzowski Jr & Hieftje 2001). Thus, inaccurate relative ion peak intensities can affect  $E_{com50}$  calculations and hinder structure identification studies (Hall et al. 2012).

Hill et al. used the relationship between RF voltage and relative ion transfer efficiency (RITE) of precursor ions to correct the peak intensities of precursor and product ions in CID spectra obtained at different RF voltages. The relative ion transfer efficiency of an ion at any RF voltage is defined relative to its peak intensity at the RF voltage that produced the maximal response. They were able to successfully correct the spectra of a compound determined at different RF voltages to a common profile. However, they did not determine whether the ion transfer efficiencies of precursor ions, which were used to construct the RF voltage-RITE relationships, were the same as those for product ions of the same  $m/z$  value. Furthermore, a single correction algorithm determined at a collision energy of 11 eV was used to correct CID spectra obtained from 1 -71 eV, which assumes similar RITE across all collision energies and RF voltages. Increased random direction kinetic energy of product ions acquired through collisions with the inert gas may lead to differences in product ion RITE which may require a different set of RF voltage – RITE relationships. Lastly, it was unclear whether the correction algorithm developed by Hill et. al. was a function of ion  $m/z$  or elemental composition.

In the current study we examined the effects of RF voltage on product ions produced within a hexapole collision cell at multiple RF voltages and collision energies. We also determined the

effects of RF voltage and collision energy on the RITE of a larger set of precursor ions. A statistical evaluation was used to determine the optimum RF voltage to be used in CID/Ecom<sub>50</sub> analyses. Precursor and product ions with similar  $m/z$  values were found to have similar RITEs at an RF voltage of 180 V. Furthermore, RITE as a function of RF voltage at four different collision energies was found to be similar at an RF voltage of 180 V. This allows for the construction of a single curve for correcting both precursor and product ion intensities at that RF voltage. Our results have implications for metabolomics analyses, especially for users relying on RF – only ion guide instruments.

## **Experimental**

### **Materials**

Aniline, benzimidazole, 8-hydroxyquinoline, formamide, glycine, imidazole, paclitaxel, strychnine and HPLC grade trifluoroacetic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Terbutaline sulfate was purchased from United States Pharmacopeia (Rockville, MD, USA). Reserpine (609 Da) was purchased from Ciba-Geigy (St. Louis, MO, USA). Reagent grade water (resistivity 18.2 MΩ-cm) was produced using a Barnstead Nanopure system from Thermo Scientific (Asheville, NC, USA). HPLC grade acetonitrile (99.9%) was purchased from Fisher Scientific (Pittsburg, PA, USA).

### **Reagent Preparation**

Stock solutions of aniline, 8-hydroxyquinoline, benzimidazole, formamide, glycine, imidazole, reserpine, strychnine and terbutaline were prepared at 500 µg/mL in water/acetonitrile (1:1). Stock solutions of paclitaxel were prepared at 1000 µg/mL in water/acetonitrile (1:1). Five mL test samples were prepared at appropriate concentrations either individually or as mixtures from the appropriate stock solutions in 0.01% (v/v) trifluoroacetic acid in water/acetonitrile (1:1).

### **Instrumentation**

Standard solutions of test compounds were analyzed on a Qtof-2 (Waters Associates, Milford, MA) mass spectrometer operating in positive ion mode. All ion optics parameters were set in accordance with the instrument manual and held constant throughout the study. Test samples were infused into the electrospray ionization source using a Hamilton syringe pump (Model # 55-2222, Hamilton Company, Reno, NV, USA) at 5 µL/min. Nitrogen was used as the nebulizer gas at approximately 100 L/h and desolvation gas at 250 L/h on the Qtof-2. The cone

gas was set at a flow rate of 50 L/h. The cone voltage was set to 20 V and the source temperature 120° C. The capillary voltage was set to 3.2 kV. Helium and nitrogen were used as collision gases at 12 psi head pressure on the Qtof-2 (estimated at  $6 - 10 \times 10^{-3}$  mbar collision cell pressure). An acceleration voltage of 200V was used. The MCP detector was set to 2250 V and the TOF flight tube to 9.1 kV. The desolvation gas temperature was set to 120 C. The Qtof-2 uses a quadrupole mass filter in tandem with a hexapole collision cell and a time-of-flight mass analyzer. The hexapole collision cell uses six stainless steel rods 160.5 mm in length arranged in a circle 9 mm in diameter, and 2 mm entrance and exit slits. (Hill et al. 2012). Masslynx 4.1 (Waters Associates, Milford, MA) was used to process mass spectral data.

#### **Effect of RF voltage and collision energy on relative ion transfer efficiency in helium**

Initial experiments were performed to evaluate the effects of RF voltage and collision energy on the RITE of 10 precursor ions. In these experiments helium was used as the collision gas in order to minimize precursor ion fragmentation. The  $[M+H]^+$  of each test compound was isolated in the quadrupole mass filter and evaluated in the hexapole collision cell at 2 eV intervals from 1 eV to 71 eV on a Qtof-2 mass spectrometer at RF voltages of 90 V, 120 V, 180 V and 300 V (peak to peak voltage). The RITE of each precursor ion (at each collision energy) was calculated as the respective ion's peak intensity divided by the peak intensity of the ion at the collision energy that gave the greatest response at each RF voltage.

#### **Effect of RF voltage and collision energy on relative ion transfer efficiency in nitrogen**

We next used collision energies of 11, 21, 31 and 41 eV in order to study the effects of RF voltage on the RITE of precursor and product ions. The  $[M+H]^+$  of each of the 10 test compounds was isolated in the quadrupole mass filter and evaluated in the collision cell using nitrogen as the collision gas. For each of the four collision energies, the collision cell RF voltage

was incrementally changed in 12 V intervals from 12 V to 180 V, in 30 V intervals from 180 V to 300 V, and in 60 V intervals from 300 V to 600 V (peak to peak voltage). The CID spectrum for each precursor ion was determined at each of the four collision energies and at each RF voltage. The relative ion transfer efficiency as a function of RF voltage was calculated as the ratio of the ion, either precursor or product, at each RF voltage to the ion's peak intensity at the RF voltage with the highest peak intensity. Ion peak intensities were corrected to their theoretical maximum by dividing the experimentally determined value with the RITE of the ion at the RF voltage used for the analysis (see online resource Figure S1).

#### **Determination and normalization of $E_{com50}$ values of protonated test compounds at multiple RF voltages**

Solutions of the test compounds, with the exception of formamide, were prepared at appropriate concentrations as described above. The samples were analyzed individually or as mixtures on the Qtof-2 mass spectrometer at an RF voltage of 180 V at every other nominal collision energy from 1 to 71 eV using nitrogen as the collision gas. At an RF voltage of 180 V, the CID spectrum at each collision energy was corrected for differences in RITE by linear interpolation using the 11 eV precursor ion response curve. The total ion current at each collision energy was calculated as the sum of the precursor and product ions intensities. The RITE of the total ion current as a function of collision energy was calculated as the ratio of the total ion current at each collision energy to the total ion current at the collision energy with the highest peak intensity. Ion peak intensities in CID spectra obtained at RF voltages of 84 and 300 V were also adjusted by interpolating RITE between collision energies using collision energy specific RITE versus  $m/z$  curves for precursor and product ions.

The survival yield (SY) of a precursor ion at any particular collision energy was calculated as the ratio of the precursor ion peak intensity to the sum of precursor and product ion intensities at that collision energy. The CE<sub>50</sub>, defined as the collision energy where 50% of the precursor ion is dissociated, was calculated from the CID spectra at collision energies that resulted in SY values between 80 % and 20 %. The center of mass energy is the theoretical amount of energy absorbed by a precursor ion upon impacting a collision gas molecule. It is calculated using equation (1).

The Ecom<sub>50</sub> value, defined as the energy required to fragment 50% of the precursor ion, for each test compound was calculated from the CE<sub>50</sub> using equation (1) as described by Hill et al (Hill et al. 2012).

### **Comparison of CID spectra after ion peak intensity adjustment**

CID spectra were obtained in triplicate at RF voltages 84 V, 180 V and 300 V. The intensities of the ion peaks in the SY20 (20% precursor ion yield) spectra were corrected as described in the previous section. Each spectrum was normalized to reflect the same mass intensity pairs, with a peak intensity of zero if a certain mass was not detected at a particular RF voltage. The coefficient of determination ( $r^2$ ) was calculated between the 180 V and 84 V spectra, 300 V and 180 V spectra and 300 V and 84 V spectra for all replicates using the root mean square function in Microsoft Excel (2010). The final  $r^2$  value for each comparison was the mean of all the replicates. The standard deviations were calculated by the 'stdev' Excel function.

### **Statistical analysis of the relative ion transfer efficiency as a function of m/z at multiple RF voltages**

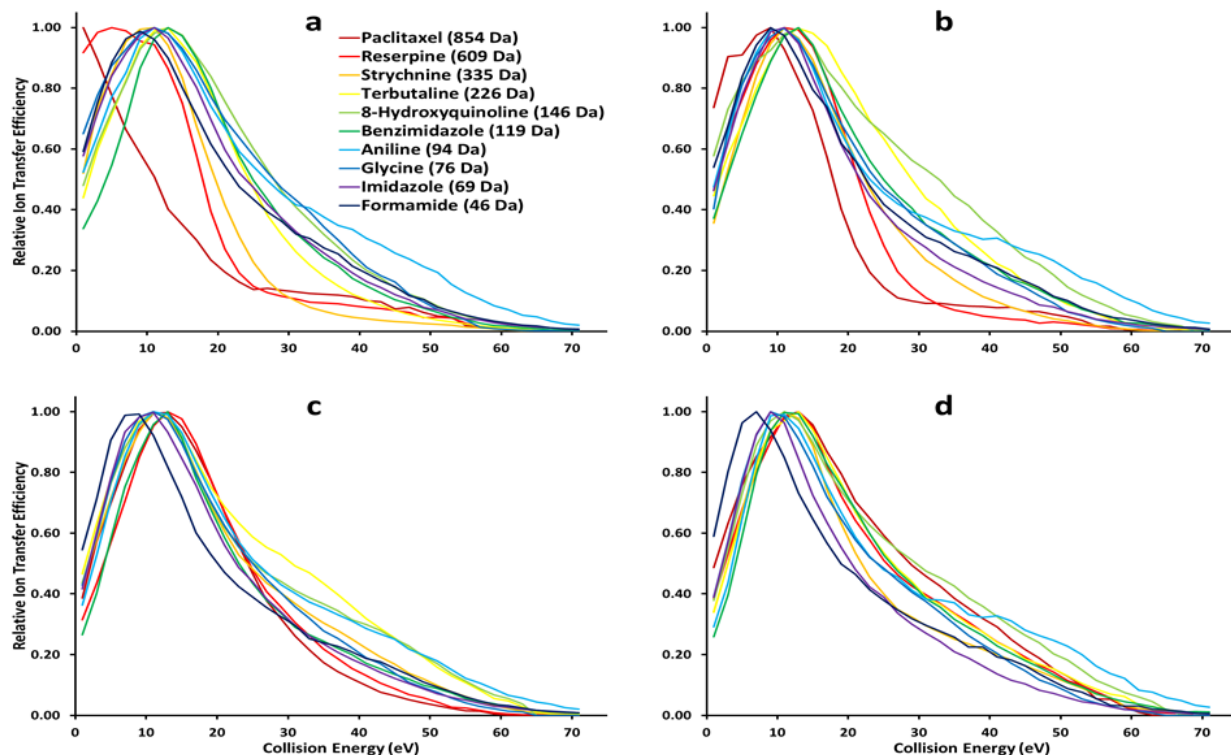
The data for the precursor and product ion RITE versus m/z at each of the 4 collision energies were fitted to different order polynomials using SAS software (SAS and all other SAS Institute Inc. products or service names are registered trademarks or trademarks of SAS Institute Inc., Cary, NC, USA.). For each data set a 6<sup>th</sup> order polynomial gave the highest coefficient of



determination and was therefore chosen as the representative equation for comparisons of each RITE versus  $m/z$  relationship at each RF voltage. The 31 eV precursor RITE versus  $m/z$  data was excluded from the comparison since only six of the ten precursor ions survived at that collision energy. The resultant equation for the RITE versus  $m/z$  curves of the precursor and product ions determined at different collision energies at a common RF voltage were compared one to the other in a pairwise manner using the F test. A probability of the null hypothesis of  $>0.005$  after applying the Bonferroni correction (Dunn 1961) was used to determine significance.

## Results and Discussion

The RITE of the  $(M+H)^+$  of each test compound as a function of collision energy was measured using helium as the collision gas at RF voltages of 90 V, 120 V, 180 V and 300 V (Figure 1). Since the peak intensity of the precursor ion entering the collision cell could not be measured with our instrument, relative ion transfer efficiencies are reported. Except for paclitaxel ( $m/z$  854) and to some extent reserpine ( $m/z$  609) at an RF voltage of 90 V, RITEs increased with collision energy up to approximately 11-13 eV after which a steady decline in signal was observed. Even at a collision energy of 70 eV, none of the test compounds produced significant fragment ions in helium. Thus, the decrease in precursor relative ion peak intensity above collision energies of 11-13 eV seems most likely due to increased scatter in the collision cell with increasing collision energy.



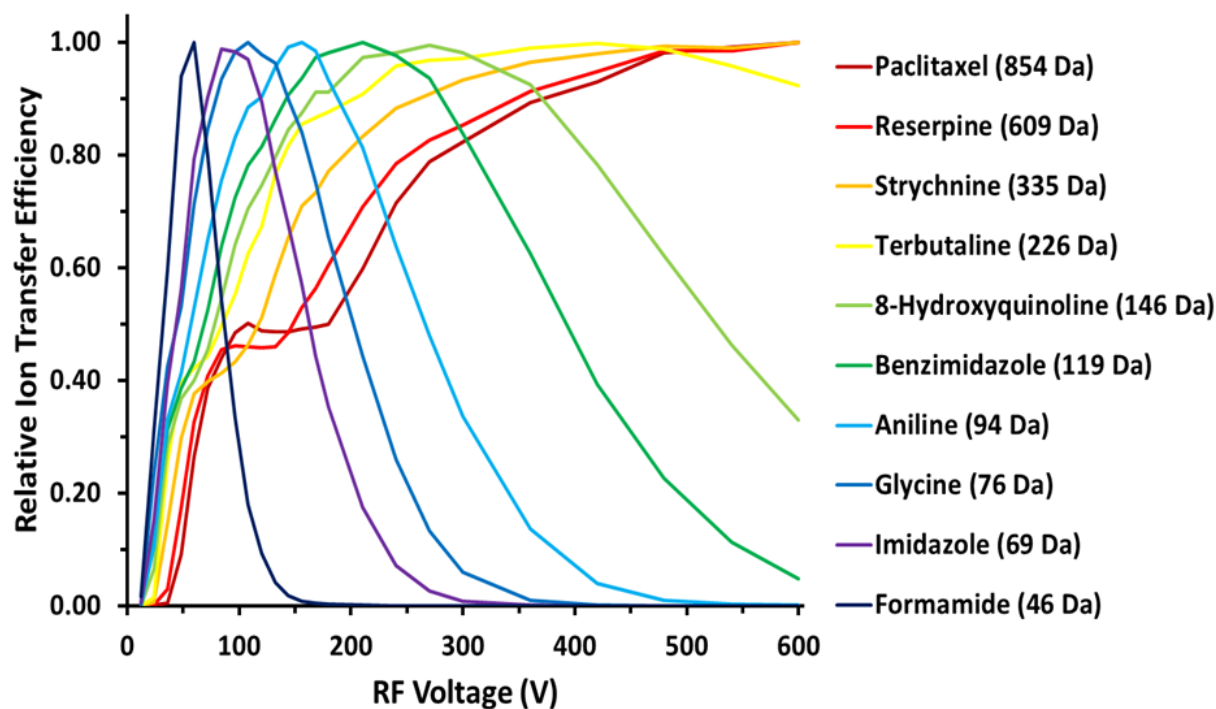
**Fig 1** Relative ion transfer efficiency as a function of collision energy (1-71eV) for the precursor ions of ten compounds at RF voltages of a) 90 V, b) 120 V, c) 180 V, d) 300 V using helium as the collision gas. Number of replicates for each compound ( $n \geq 2$ ). Average RITE standard deviation = 0.0097

Figure 1 clearly shows that the RITE varies with collision energy. However, as observed for paclitaxel, the  $m/z$  of the precursor ion also influences the overall shape of these curves, especially at RF voltages of 90 and 120 V. For example, at an RF voltage of 90 V (Figure 1a), paclitaxel does not show the typical increase in signal from 0-12 eV. Instead, paclitaxel shows only a steady decline with increasing collision energy. At an RF voltage of 120 V, however, paclitaxel shows an increase in RITE up to approximately 12 eV. In addition, at an RF voltage of 90, the RITEs for paclitaxel, reserpine and strychnine deteriorate to less than 20% at collision energies above approximately 25 eV, whereas  $m/z$  ions of 226 and below have considerably higher RITEs above 25 eV. In general, ions with  $m/z$  values greater than 226 have dissimilar RITE versus CE profiles as compared to ions with lower  $m/z$  values at RF voltages of 90 V and 120 V (Figure 1 a, b). These

results indicate that the effects of RF voltage on the RITE vs CE curves depend to some extent on the  $m/z$  of the ion. However, the RITE profiles of the precursor ions appear more similar at RF voltages of 180 V and 300 V. That is, loss in ion transfer efficiency due to scatter appears to be similar among precursor ions at these two RF voltages.

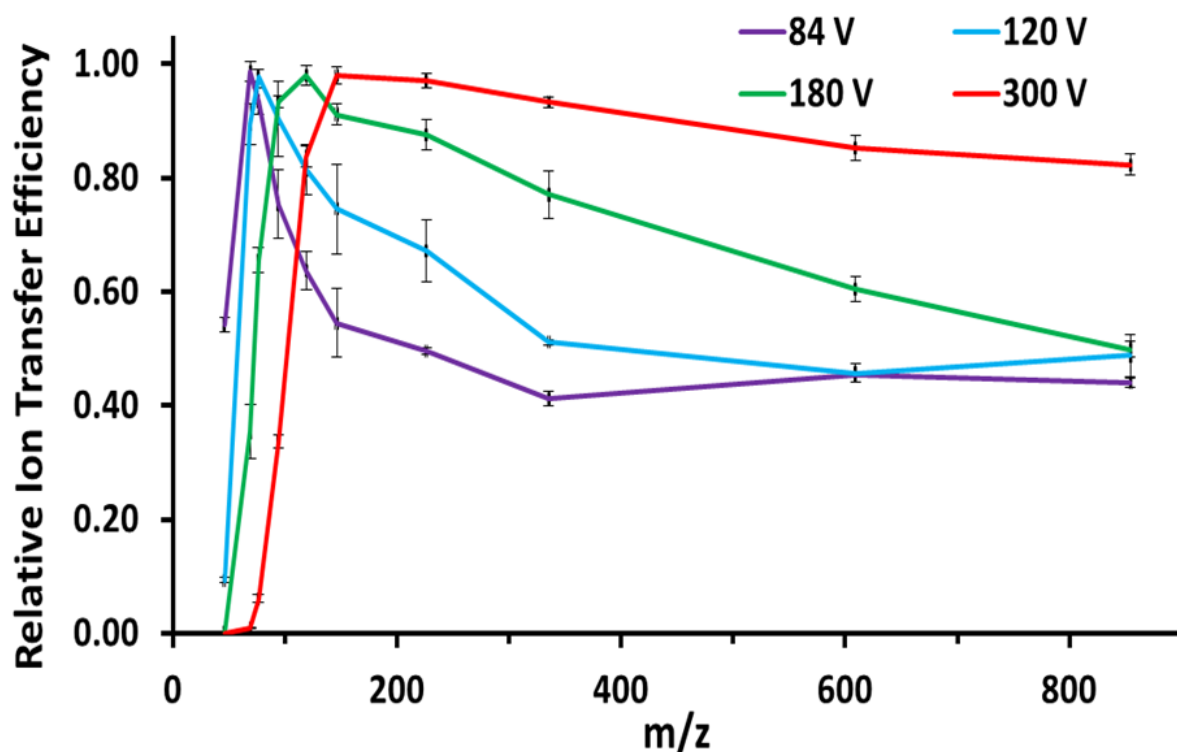
In previous work, Hill, et al. reported RITE as a function of RF voltage (0-600V) at a collision energy of 5 eV using a hexapole/TOF instrument and argon as the collision gas. In that study, precursor ions with lower  $m/z$  values (<146 Da) were found to be most efficiently transferred at lower RF voltages (120 V – 180 V) with the exception of formamide which had a peak ion transfer efficiency at 60 V. In contrast, precursor ions with higher  $m/z$  values (>146 Da) were most efficiently transferred at RF voltages between 300 V and 600 V, although reasonable RITE efficiencies (~50% - 90%) were obtained at an RF voltage of 180 V. As shown in Figure 1 using helium, the collision energy that gives the highest sensitivity in the Qtof-2 mass spectrometer is approximately 11 eV. The instrument manual suggests that a collision energy of approximately 10 eV provides the highest sensitivity. However, compounds with low  $E_{com50}$  values may begin to fragment below 11 eV when a collision gas such as argon is used.

Since the amount of energy absorbed by a compound at a specific collision energy is proportional to the mass of the collision gas, we next measured the RITE of precursor ions as a function of RF voltage at a collision energy of 11 eV using nitrogen as the collision gas (Figure 2).



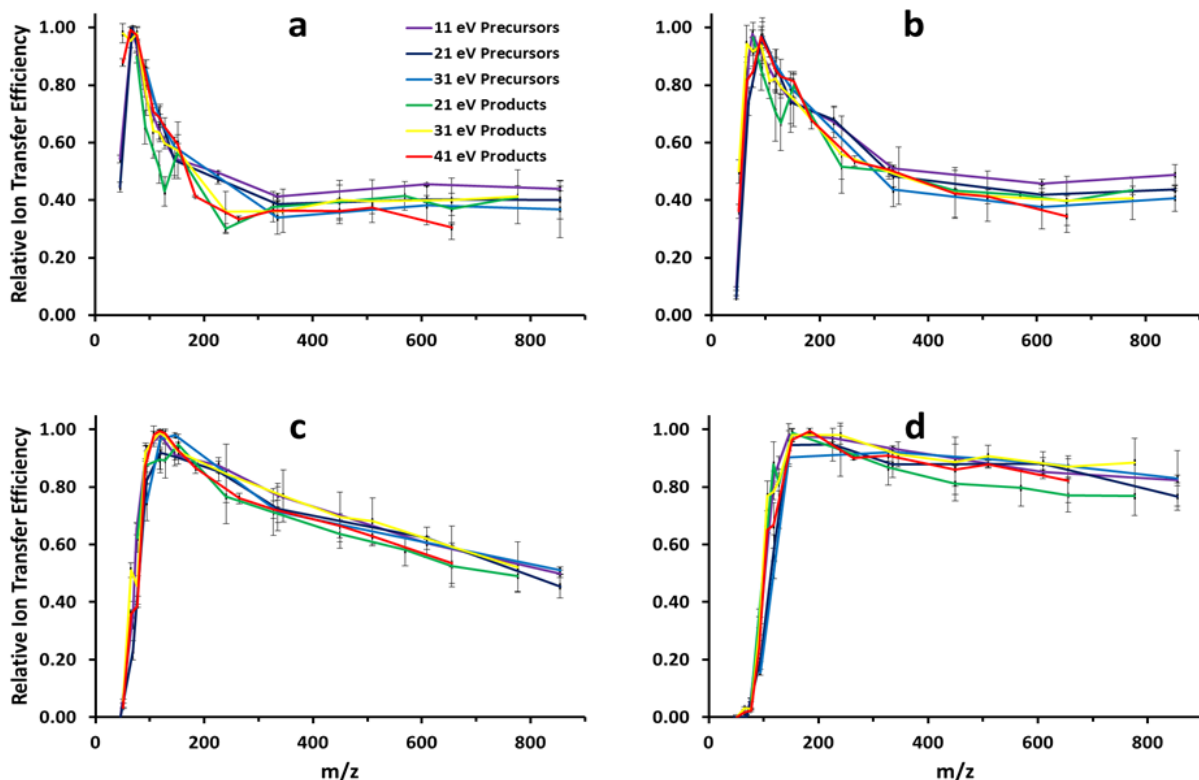
**Fig 2** Relative ion transfer efficiency as a function of RF voltage (12 – 600V) for 10 precursor ions at a collision energy of 11 eV using nitrogen as the collision gas. The RF voltage was incremented in 12 V steps from 12 V to 180 V, 30 V steps from 180 V to 300 V and 60 V steps from 300 V to 600 V. Number of replicates from each compound ( $n \geq 3$ ). Average RITE standard deviation = 0.019

A collision energy of 11 eV was chosen because fragmentation of test compounds was minimal in nitrogen at this collision energy. The results indicate that precursor ions less than  $m/z$  76 are poorly detected at RF voltages above 300 V (Figure 2). This is consistent with what was previously shown. Thus, for any given collision energy, RF voltages of 300 V and above would significantly affect  $E_{com50}$  determinations and CID spectral profiles if major product ions below  $m/z$  76 were present in the spectrum. Although an RF voltage of 120 V would improve the recovery of lower  $m/z$  ions (i.e. formamide, 46 Da), the RITE profiles as a function of collision energy at this voltage (Figure 1 b) are not consistent among all masses. These results indicate that an RF voltage of 180 V is a reasonable compromise for the detection of lower  $m/z$  ions ( $m/z$  46 –  $m/z$  119) while not significantly diminishing the recovery of larger  $m/z$  ions ( $>m/z$  146) at higher collision energies.



**Fig 3** Relative ion transfer efficiency as a function of  $m/z$  for the ten precursor ions listed in Figure 2. The RF voltages were 84 V, 120 V, 180 V and 300 V. Nitrogen was used as the collision gas and the collision energy was 11 eV. Number of replicates for each compound ( $n \geq 3$ ).

The RITE of precursor ions as a function of  $m/z$  at RF voltages of 84 V, 120 V, 180 V and 300 V are plotted in Figure 3. These results are similar to those previously obtained by Hill et al. (Hill et al. 2012) using argon as the collision gas at an RF voltage of 180 V (Online Resource Figure S2).

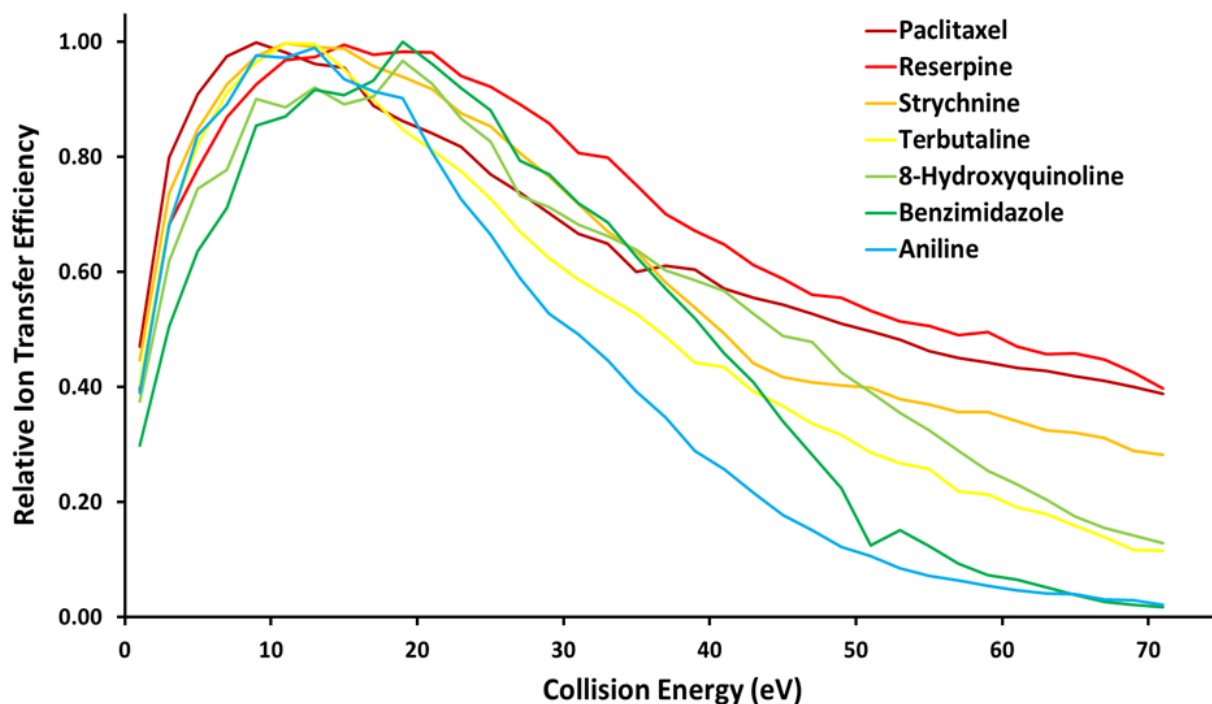


**Fig 4** Relative ion transfer efficiency of precursor and product ions at RF voltages a) 84 V, b) 120 V, c) 180 V and d) 300 V at collision energies of 11 eV, 21 eV, 31 eV and 41 eV using nitrogen as the collision gas. Number of replicates for each compound ( $n$ )  $\geq 3$ .

It is possible that the relationship between RF voltage and RITE of product ions is different from that of precursor ions due to higher random-direction kinetic energy and ion scatter. Thus, the RITE at RF voltages of 84, 120, 180 and 300 V were determined for both precursor and product ions in positive ion mode at collision energies of 11, 21, 31 and 41 eV using nitrogen as the collision gas (Figure 4). Ions with  $m/z$  values  $< 46$  fall below our mass detection limit and thus were not included in these correction curves. The results of a pairwise statistical comparison of these curves indicated that the RITE versus  $m/z$  relationship between precursor and product ions at different collision energies are the same at an RF voltage of 180 V (Online Resource Tables T1 – T4). Furthermore, similar  $m/z$  product ion peaks from different precursors also had comparable RITE's at an RF voltage of 180 V (Online Resource Figure S3). We found

significant differences in the RITE versus  $m/z$  curves at the other RF voltages tested, however, it was unclear whether product ions had a lower ion transfer efficiency as compared to precursor ions. The statistical analysis did, however, indicate that a single correction curve at an RF voltage of 180 V could be used to correct for differences in RITE as a function of mass regardless of whether the ion is a precursor or a product ion. We tested the accuracy of the 180 V RF precursor correction curve using a test set of 5 additional compounds (Apramycin (504 Da), Glycocholic acid (466 Da), Testosterone (289 Da), Amino adipic acid (162 Da) and N-Acetylputrescine (131 Da)) and their fragment ions by comparing the experimentally determined RITE of these ions to their predicted RITE values (Online Resource Figure S4). The RITE of these ion calculated from the derived RITE- $m/z$  correction curve were within 2 standard deviations of the mean experimental RITE values.





**Fig 5** Relative ion transfer efficiency as a function of collision energy (1-71 eV) of total ion current (sum of precursor and product ion peak intensities) at an RF voltage of 180 V using nitrogen as the collision gas. For each of the seven compounds total ion current was corrected using the 11eV precursor 180 RF voltage correction curve (single correction) shown in Figure 3. Number of replicates for each compound ( $n \geq 3$ ). Average RITE standard deviation = 0.0290

A single correction curve based on the RITE vs  $m/z$  relationship of precursor ions at 11 eV (Figure 3) was used to adjust precursor and product ion peak intensities in the CID spectra of aniline, benzimidazole, 8-hydroxyquinoline, reserpine, strychnine, paclitaxel and terbutaline obtained at RF voltages of 180 V using every other collision energy from 1 – 71 eV. The corrected RITE of precursor and product ions was determined by interpolation (as shown in Online Resource Figure S1) between appropriate  $m/z$  values. The peak intensity of product ions generated by formamide (46 Da), imidazole (69 Da) and glycine (76 Da) were not included as their fragments fall below our detection limit as explained above. Figure 5 shows that the corrected relative total ion current of each test compound analyzed in nitrogen did not degrade as sharply with increasing collision energy as was observed when using helium as the collision gas.

One possible explanation for these results is that collisional cooling improves transmission efficiency by dampening the trajectory of ions, thereby bringing them closer to the center of the hexapole. Ions thus experience a loss in both radial and axial velocity as a result of collision with the collision gas (Douglas & French 1992). These results are consistent with the findings of Guzowski and Heiftje (Guzowski Jr & Hieftje 2001) who showed that heavier collision gases were more effective at focusing the ion beam and Tanner and Baranov (Tanner & Baranov 1999) who observed that collisional cooling is affected by the ratio of the mass of the collision gas to the mass of the analyte. Since collisional cooling plays an important role in focusing the ion beam, the effect of collision cell gas pressure on  $E_{com50}$  warrants further investigation.

It was previously shown that CID spectra and  $E_{com50}$  values for benzimidazole obtained at different RF voltages could be adjusted to similar values using a single precursor RITE versus  $m/z$  curve for each RF voltage. Table 1 and 2 show the comparison of  $E_{com50}$  values and cross correlation matching of CID spectra (respectively) obtained at RF voltages of 84 V, 180 V, and 300 V for aniline, benzimidazole, 8-hydroxyquinoline, paclitaxel, reserpine, strychnine, and terbutaline.

**Table 1** Ecom<sub>50</sub> values (eV) calculated before and after normalization from CID spectra obtained at RF voltages of 84 V, 180 V and 300 V using every other collision energy from 1 - 71 eV in nitrogen.

	Ecom <sub>50</sub> Uncorrected					Ecom <sub>50</sub> 11 eV Precursor Ion Correction					Ecom <sub>50</sub> Multiple Curve Correction				
Compound	84 V	180 V	300 V	Average	Stdev	84 V	180 V	300 V	Average	Stdev	84 V	180 V*	300 V	Average	Stdev
Aniline	4.89	5.16	6.18	5.41	0.68	5.04	4.87	4.83	4.91	0.11	4.94	4.87	5.20	5.00	0.17
Benzimidazole	6.05	6.54	7.79	6.79	0.90	6.25	6.09	5.76	6.03	0.25	6.25	6.09	6.44	6.26	0.17
8-Hydroxyquinoline	4.75	4.80	4.93	4.83	0.09	4.84	4.74	4.83	4.80	0.06	4.76	4.74	4.76	4.75	0.01
Terbutaline	1.71	1.78	1.76	1.75	0.04	1.74	1.79	1.74	1.76	0.03	1.75	1.79	1.75	1.76	0.02
Strychnine	3.24	3.32	3.38	3.31	0.07	3.37	3.47	3.33	3.39	0.07	3.49	3.47	3.31	3.42	0.10
Reserpine	1.84	1.62	1.82	1.76	0.12	1.83	1.73	1.86	1.81	0.07	1.80	1.73	1.82	1.78	0.05
Paclitaxel	0.37	0.35	0.37	0.36	0.01	0.37	0.37	0.37	0.37	0.00	0.38	0.37	0.36	0.37	0.01

\* Normalized using 11 eV Precursor Correction

Without ion peak intensity adjustments aniline, benzimidazole, and to a lesser extent, reserpine had divergent  $E_{com50}$  values and poor cross correlation of CID spectral profiles between RF voltages; however, these values for the other test compounds were similar between RF voltages. The relative intensities of the precursor and product ions in the CID spectra of these compounds were initially adjusted using the respective 11 eV precursor RITE vs  $m/z$  curve for each RF voltage as previously described. This correction resulted in a decrease in the variability of the  $E_{com50}$  values and CID spectral cross correlations for aniline, benzimidazole, and reserpine (Table 1 and 2) between the RF voltages used. Further adjustment of the ion peak intensities using multiple precursor, product, and collision energy RF voltage specific RITE versus  $m/z$  curves for the aniline and benzimidazole CID spectra determined at RF voltages 84 V and 300 V resulted in a similar decrease in the variability of the  $E_{com50}$  and CID spectral profiles between data collected at different RF voltages.

**Table 2** Coefficient of determination values of CID spectra obtained at RF voltages of 84 V, 180 V and 300 V. CID spectra for all compounds at each RF voltage was obtained at collision energies corresponding to approximately 20% survival yield after RITE normalization at RF 180 V.

Compound	r <sup>2</sup> Uncorrected						r <sup>2</sup> 11 eV Precursor Ion Correction					r <sup>2</sup> Multiple Curve Correction				
	Collision Energy	84 vs 180	84 vs 300	180 vs 300	Average	Stdev	84 vs 180	84 vs 300	180 vs 300	Average	Stdev	84 vs 180*	84 vs 300	180* vs 300	Average	Stdev
<b>Aniline</b>	27 eV	0.98	0.63	0.73	0.78	0.18	1.00	1.00	1.00	1.00	0.00	1.00	0.91	0.92	0.94	0.05
<b>Benzimidazole</b>	39 eV	0.71	0.25	0.76	0.57	0.28	0.98	0.77	0.87	0.87	0.11	0.97	0.99	0.96	0.97	0.02
<b>8-Hydroxyquinoline</b>	35 eV	0.99	0.93	0.96	0.96	0.03	0.99	0.93	0.96	0.96	0.03	0.99	0.70	0.72	0.81	0.16
<b>Terbutaline</b>	19 eV	0.97	0.99	0.99	0.98	0.01	0.98	1.00	0.96	0.98	0.02	0.97	1.00	0.97	0.98	0.02
<b>Strychnine</b>	51 eV	0.93	0.85	0.97	0.92	0.06	0.91	0.99	0.87	0.92	0.06	0.99	0.76	0.79	0.85	0.13
<b>Reserpine</b>	45 eV	0.59	0.99	0.58	0.72	0.23	0.81	0.99	0.81	0.87	0.10	0.81	0.98	0.82	0.87	0.10
<b>Paclitaxel</b>	15 eV	0.94	0.99	0.97	0.97	0.03	0.95	0.94	0.98	0.96	0.02	0.97	0.99	0.97	0.97	0.01

\* Normalized using 11 eV Precursor Correction

Additionally, the cross correlation comparison between the CID spectra of aniline and benzimidazole determined at different RF voltages approached 1.0 (Online Resource Figures S5 and S6), while it improved drastically for reserpine. These data are consistent with what is observed in Figures 3 and 4, i.e. that the RITE is less variable for ions greater than  $m/z$  150, whereas there is greater variability among ions with  $m/z$  values  $<150$ . Most of the heavier compounds ( $>146$  Da) used in this study generate major product ions that are greater than 150  $m/z$  and therefore there is very little difference in the RITE between ions. Even though the magnitude of the RITE values of these ions may vary between RF voltages, the constant RITE between ions ensures relative ion peak intensities remain the same between RF voltages. Berendsen et al. (Berendsen et al. 2013) show that precursor ions in the 100 – 200 Da range are most likely to produce major product ions with masses of approximately 100 Da. Heavier precursor ions, in the 200-300 Da range and above, have major product ions between 140 - 170 Da. Thus, the correction curves presented in our study may have wider applicability than indicated by the ten compounds used. The precursor and product ions in the CID spectra of benzimidazole and aniline have  $m/z$  values less than 150 and therefore vary greatly in their RITEs. Additionally, RITE versus  $m/z$  relationships vary between RF voltages. This results in considerable differences in relative ion peak intensities in CID spectra determined at different RF voltages. However, the ion peak intensity adjustments normalize these spectra to a common profile.

Theoretical models developed by Knyazev and Stein (Knyazev & Stein 2010) suggest that product ions may have lower absolute ion transfer efficiencies as compared to precursor ions of similar  $m/z$  due to additional kinetic energy acquired during CID. The calculated precursor ion efficiency was not included in their results, thus the magnitude of this difference could not be

ascertained. If the absolute intensity of product ions is lower than precursor ions, a new correction algorithm that accounts for these differences would have to be developed. However, if the maximum absolute ion transfer efficiencies of all ions are the same, RITE corrected CID spectra will reflect true relative ion abundances. Nevertheless, our results suggest that our approach appears to normalize Ecom50 and CID spectral profiles which can be beneficial for structure identification and untargeted metabolomics analyses.

RF-only multipole ion guides are common in mass spectrometry (Giles et al. 2004). Much of the work relating to their effects on ion transfer efficiency has so far been confined to theoretical analyses and largely ignored in metabolomics studies. Commonly used metabolomic databases such as HMDB and METLIN contain CID spectra acquired using instruments with RF-only ion guides as collision cells. The HMDB MS/MS database was constructed using a Waters Micromass Quattro Triple Quad mass spectrometer which has an RF voltage range of 0 – 500 V (Wishart et al. 2009). The METLIN database was constructed using an Agilent 6538 Q-TOF which has an RF voltage range of 10 - 550 V (Tuatenhahn et al. 2012). Neither database corrects for the effects of RF voltage on fragment abundance, nor is it clear what RF voltage was used. Although a complete theoretical treatment of RF only ion guides is beyond the scope of the work presented here, Gerlich (Gerlich 2004) and Szabo (Szabo 1986) have reviewed this subject. RF based mass discrimination in CID spectra has also been observed in instruments using stacked ring ion guides (Hill et al. 2012).

From an experimental standpoint, the RF correction described here would increase the accuracy of survival yield and Ecom<sub>50</sub> calculations and would make CID spectra comparisons more reliable. An RF voltage analysis with standards covering a broad mass range (as shown in Figure 2) could be performed on any instrument using a RF only ion guide collision cell to

determine its RITE as a function of  $m/z$ . The RF voltage is just one of many instrument parameters and experimental conditions that can potentially affect CID spectra. Other factors that warrant investigation include the RF frequency, gas pressure, type of detector and detector voltage. The effects of RF frequency on ion abundance are of particular interest as it plays an important role in the mass range transmitted by RF-only ion guides (Guzowski Jr & Hieftje 2001).



## Concluding Remarks

The results of this study highlight the effect of collision cell RF voltage, collision energy and collision gas on CID spectra in a hexapole collision cell. We show that these experimental conditions affect the relative ion peak intensities of CID spectra that contain significant product ions with  $m/z$  values less than 150. In order to more accurately compare the CID spectral data of these compounds with spectra determined in different laboratories using multipole/TOF mass spectrometers it is important to correct ion peak intensities to their actual relative abundance. This is especially important if low mass fragment ions are a significant component of the CID spectrum. We demonstrate that estimates of the RITE of ions of different  $m/z$  values can be used to adjust the ion peak intensities to common values across these experimental variables. Our results indicate a collision cell RF voltage of 180 V allows adequate sensitivity across a wide mass range on our instrument and renders CID spectra and  $E_{com50}$  values amenable to a single, straightforward correction. The RF voltage normalization procedure described in this study is relevant to several metabolomics databases currently in use. An investigation of other instrument parameters and experimental conditions would further improve our ability to standardize and compare CID spectra collected among different laboratories.

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## **Compliance with Ethical Standards**

The authors declare that they have no conflict of interest. This article does not contain any studies with human participants or animals performed by any of the authors.

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