*Research Article*

# Optimization of Resolving Power, Fragmentation, and Mass Calibration in an Orbitrap Spectrometer for Analysis of 24 Pesticide Metabolites in Urine

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Mass spectrometer parameters such as Resolving Power, type of fragmentation, and mass calibration mode were optimized in the analysis of 24 pesticide metabolites in human urine using Ultra-High Pressure Liquid Chromatography coupled to Orbitrap High-Resolution Mass Spectrometer (UHPLC-HRMS). The best results were achieved with a Resolving Power of 25,000 FWHM and by applying Collision Induced Dissociation fragmentation mode (40 eV).

## 1. Introduction

The ever growing number of chemicals being used, such as pesticides, care products, UV filters, parabens, and so on, has an impact on the environment and therefore on humans, especially in vulnerable populations [1]. To protect consumers from such contaminants, an evaluation of the exposure to these chemicals and the subsequent risk assessment is necessary. The National Report on Human Exposure to Environmental Chemicals in the United States [2] and the Human Biomonitoring Report of Environmental Chemicals in Canada [3] are both examples of biomonitoring programs that assess the exposition of a population to environmental chemicals over time. The European Environment and Health Strategy also encourages the adoption of human biomonitoring studies across Europe [4].

Some pesticide metabolites are biomarkers of pesticide exposure. These metabolites are present in urine at concentrations of few ng mL$^{-1}$ [2], and consequently metabolite determination requires sensitive and selective analytical methods. The usual analytical technique for polar metabolites in human biomonitoring studies is the LC-MS/MS [5]. However, the introduction of the high-resolution (>10,000 FWHM) mass spectrometers, which allow to obtain mass accuracies lower than 5 ppm, such as Q-TOF or Orbitrap, has allowed the implementation of combined quantitative target and postrun target analytical strategies for comprehensive determination of pesticides and other emerging contaminant metabolites [6].

Over the last few years, liquid chromatography coupled to Orbitrap high-resolution mass spectrometry (LC-HRMS) has been applied in human biomonitoring studies [6]. Recently, Cortejade *et al.* (2016) developed an analytical method for the targeted screening and multiresidue quantification of 38 contaminant metabolites in urine, including 12 pesticides, a pesticide metabolite (tributyl phosphate), and other compounds of different families [7]. Likewise, Roca *et al.* (2014) developed an analytical method that combined the quantitative target analysis of urinary metabolites of pesticides with a retrospective analysis using liquid chromatography coupled to HRMS. In this study, the main factors governing the ion-source ionization were optimized [6]. In addition, *López et al.* (2016) developed a retrospective analytical methodology for the analysis of pesticide metabolites in urine by LC-HRMS [8].

In the existing literature on application of HRMS to food and feed contaminants [9–14] and in the field of human biomonitoring [6, 15–21], the emphasis so far has mainly been the detectability of the analytes based on retention time and the exact mass of the most abundant analyte ion and on quantitative determination. However, Resolving Power (R) and mass calibration should be further investigated in order to avoid interferences and improve mass accuracy. Likewise, the selection of the fragmentation mode (High-energy Collision Dissociation, HCD, or Collision Induced Dissociation, CID) and their optimization could improve the sensitivity of the confirming ions (fragments).

How much Resolving Power is necessary to apply to a specific problem should be a frequent analytical question [22]. Orbitrap mass spectrometers allow a wide range of Resolving Powers. Theoretically, a higher Resolving Power provides a better resolution of analytes from isobaric interferences present in complex matrices such as urine. However, in Orbitrap, greater Resolving Power requires longer measurement time. A higher Resolving Power leads to the monitoring of fewer data points per time unit [23]. Therefore, for quantitative analysis, the Resolving Power for each matrix-analyte combination should be selected taking into account the isobaric interferences and the number of data points required for provide good peak shape. The Resolving Power has been studied in the literature for veterinary drugs in food and animal samples [22] as well as for other residues and contaminants in food and feed [24].

In order to obtain a suitable mass accuracy (< 5 ppm) in an Orbitrap spectrometer, a proper mass calibration must be employed. External mass calibration is performed previously to the analysis by direct infusion of a mix of compounds with known masses; the experimental m/z values obtained are then corrected with the theoretical m/z values in order to fit the accuracy of the analyzer. Until now, external mass calibration in Orbitrap has been widely employed in biological samples, food, and feed [6, 7, 11, 12, 18, 20]. However, as Leendet *et al.* (2015) have pointed out "improper external mass calibration can lead to large systematic errors in mass measurements" and "external mass calibration range must include the mass range of interest" [25]. Another strategy is the use of internal mass calibration, which is achieved by introducing a compound with a known theoretical m/z value (lock mass) during the analysis. The comparison between the theoretical and experimental errors is used to normalize the m/z values of the rest of peaks [26]. Thus, Strano-Rossi *et al.* (2015) successfully determined stimulants and drugs in food supplements using internal mass calibration in an Orbitrap system [27]. Internal calibration is recommended to avoid drifts of the external calibration over time [25].

Unlike conventional quadrupole (QqQ) instruments, Orbitrap Exactive™ users do not implement a compound-specific fragmentation optimization. In Exactive™, two all ion fragmentation (AIF) modes are allowed: HCD and CID [28]. In HCD, the ions are fragmented in a collision cell using $N_2$ as a collision gas, while CID allows the ions dissociation through interaction with neutral target species. Consequently, some authors have studied the fragmentation, optimizing the HCD cell for different substances in biological samples, food and feed [6, 11, 12, 18, 20] in an Orbitrap detector. Optimization of HRMS (Orbitrap) fragmentation mode has also been employed in areas such as proteomics [28] or for the identification of oligosaccharides [29]. Optimization of the different types of fragmentation modes (HCD and CID) will probably improve the sensitivity of these methods.

In a previous work [6], we studied the influence of the HCD collision energy on the fragmentation of various pesticide metabolites in urine, working at 50,000 FWHM. In the present paper, we want to increase the speed of the analysis using the polarity switching function (ESI⁺ and ESI⁻ in the same injection) and to study more in depth other factors that can have a decisive influence on the sensitivity, accuracy and speed of the analysis of pesticide metabolites in urine. Consequently, the aims of the present work are to (i) study the influence of Resolving Power (R) on the signal intensity and mass accuracy of the different ions; (ii) compare the two all ions fragmentations (AIF) modes (HCD; CID); (iii) evaluate the two options for mass calibration (internal and external) to improve mass accuracy for this specific application.

## 2. Materials and Methods

This study has been developed in the framework of the DENAMIC project, which included all the required ethical approvals.

*2.1. Reagents and Chemicals.* Solvents were specific for pesticide residue analysis and of analytical grade. Acetonitrile and methanol were supplied by Scharlab (Barcelona, Spain). Acetic acid (purity 98-100%), $\beta$-glucuronidase arylsulfatase enzyme, and anhydrous sodium acetate were obtained from Merck (KGaA, Darmstadt, Germany). Deionized water was organically and biologically purified by using a Milli-Q Ultra-pure System (Millipore, Darmstadt, Germany). QuEChERS EN extraction kits, containing 4 g $MgSO_4$; 1 g NaCl; 1 g Sodium Citrate; 0.5 g Sodium Hydrogencitrate Sesquihydrate, were obtained from Agilent Technologies (Madrid, Spain).

Standards of pesticide metabolites (Table 1) were achieved. All commercial standards were of high purity and were obtained from Dr. Ehrenstorfer (Augsburg, Germany), Sigma-Aldrich (Barcelona, Spain), Cerilliant-Certificated Reference Materials (Texas, USA), Cambridge Isotope Laboratories (Massachusetts, USA), Santa Cruz Biotechnology (Heidelberg, Germany), and AccuStandard (New Haven, USA). Stock standard solutions containing 20–500 mg $L^{-1}$ of the individual compounds were prepared by weighing each compound and dissolving it in acetonitrile. Solutions were stored at $-20°C$. Multianalyte intermediate standard solutions were prepared by diluting the individual stock solutions in acetonitrile and used for preparing working mixed-standard solutions in acetonitrile: water (10:90, v/v). The concentration of the analytes in working solutions ranged from 1000 to 5000 ng·mL$^{-1}$ depending on the compound.

TABLE 1: Pesticide metabolites: diagnostic and fragment ions used for analysis.

| Class | Compound | Metabolite | Acronym | Elemental composition | Diagnostic ion | Exact mass m/z diagnostic ion (Da) | Fragment elemental composition | m/z fragment ion (Da) |
|---|---|---|---|---|---|---|---|---|
| Organophosphate insecticides | Chlorpyrifos, chlorpyrifos-methyl | 3,5,6-Trichloro-2-pyridinol | TCPy | $C_5H_2NOCl_3$ | $[M-H]^-$ | 195.91292 | - | - |
| | Parathion, methyl parathion | p-nitrophenol | PNP | $C_6H_5NO_3$ | $[M-H]^-$ | 138.01966 | $C_6H_4O_2$ | 108.02167 |
| | Pirimiphos-methyl | 2-Diethylamino-6-methyl-6-hydroxypyrimidine | DEAMPY | $C_9H_{15}N_3O$ | $[M+H]^+$ | 182.12879 | $C_7H_{12}N_3O$ | 154.09748 |
| | Diazinon | 2-Isopropyl-4-methyl-6-hydroxypyrimidine | IMPY | $C_8H_{12}N_2O$ | $[M+H]^+$ | 153.10224 | $C_4H_6NO$ | 84.04439 |
| | Coumaphos | 3-Chloro-7-hydroxy-4-methylcoumarin | CMHC | $C_{10}H_7ClO_3$ | $[M-H]^-$ | 209.00109 | $C_9H_5O_2$ | 145.02841 |
| | Fenitrothion | 3-Methyl-4-nitrophenol | MNP | $C_7H_7NO_3$ | $[M-H]^-$ | 152.03531 | $C_7H_6O_2$ | 122.03733 |
| | Dimethoate | Dimethoate | DIMET | $C_5H_{12}NO_3PS_2$ | $[M+H]^+$ | 230.00690 | $C_2H_6O_2PS$ | 124.98206 |
| | Omethoate | Omethoate | OMET | $C_5H_{12}NO_4PS$ | $[M+H]^+$ | 214.02974 | $C_4H_8O_4PS$ | 182.98754 |
| | Acephate | Acephate | AP | $C_4H_{10}NO_3PS$ | $[M+H]^+$ | 184.01917 | $C_2H_8O_3PS$ | 142.99262 |
| | Methamidophos | Methamidophos | MMP | $C_2H_8NO_2PS$ | $[M+H]^+$ | 142.00861 | - | 112.01577 |
| | Chlorethoxyphos, chlorpyrifos coumaphos, diazinon, disulfoton, ethion, parathion, phorate, phosalone, sulfotep, terbufos, azinphos-methyl, dichlorvos, dicrotophos, dimethoate, fenitrothion, fenthion, malathion, methyl parathion, trichlorfon, chlorpyrifos-methyl, methidathion, mevinphos, oxydemeton-methyl, phosmet, pirimiphos-methyl, temephos, tetrachlorvinphos, isazofos-methyl, naled | Diethyl phosphate | DEP | $C_4H_{11}O_4P$ | $[M-H]^-$ | 153.03221 | $C_2H_6O_4P$ | 125.00092 |
| | | Diethyl thiophosphate | DETP | $C_4H_{11}O_3PS$ | $[M-H]^-$ | 169.00937 | $C_2H_6O_3PS$ | 140.97807 |
| | | Dimethyl thiophosphate | DMTP | $C_2H_7O_3PS$ | $[M-H]^-$ | 140.97807 | $CH_3O_3PS$ | 125.95460 |
| | | Dimethyl dithiophosphate | DMDTP | $C_2H_7O_2PS_2$ | $[M-H]^-$ | 156.95523 | $CH_3O_2PS_2$ | 141.93174 |
| Phenoxy herbicides | 2,4-Dichlorophenoxyacetic acid | 2,4-Dichlorophenoxyacetic acid | 2,4-D | $C_8H_6O_3Cl_2$ | $[M-H]^-$ | 218.96212 | $C_6H_3OCl_2$ | 160.95664 |
| | 2,4,5-Trichlorophenoxyacetic acid | 2,4,5-Trichlorophenoxyacetic acid | 2,4,5-T | $C_8H_5O_3Cl_3$ | $[M-H]^-$ | 252.92315 | $C_6H_2OCl_3$ | 194.91767 |
| Chloroacetanilide herbicides | Atrazine | Atrazine mercapturate | ATZM | $C_{13}H_{22}N_6O_3S$ | $[M+H]^+$ | 343.15468 | $C_8H_{16}N_5S$ | 214.11209 |
| | Alachlor | Alachlor mercapturate | ALAM | $C_{19}H_{28}N_2O_5S$ | $[M+H]^+$ | 397.17916 | $C_5H_8N O_3$ | 130.04987 |
| | Metolachlor | Metolachlor mercapturate | METM | $C_{20}H_{30}N_2O_5S$ | $[M+H]^+$ | 411.19481 | $C_{15}H_{24}N O_2S$ | 282.15223 |

Table 1: Continued.

| Class | Compound | Metabolite | Acronym | Elemental composition | Diagnostic ion | Exact mass m/z diagnostic ion (Da) | Fragment elemental composition | m/z fragment ion (Da) |
|---|---|---|---|---|---|---|---|---|
| | Commercial Pyrethroids | 3-Phenoxybenzoic acid | PBA | $C_{13}H_{10}O_3$ | $[M-H]^-$ | 213.05571 | $C_{12}H_9O$ | 169.06589 |
| | Cyfluthrin | 4-Fluoro-3-phenoxybenzoic acid | FPBA | $C_{13}H_9FO_3$ | $[M-H]^-$ | 231.04629 | $C_{12}H_8OF$ | 187.05647 |
| Pyrethroid insecticides | | cis-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid | cis-DCCA | $C_8H_{10}O_2Cl_2$ | $[M-H]^-$ | 206.99850 | - | - |
| | Permethrin, cypermethrin, cyfluthrin | trans-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid | trans-DCCA | $C_8H_{10}O_2Cl_2$ | $[M-H]^-$ | 206.99850 | - | - |
| | Deltamethrin | cis-(2,2-Dibromovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid | DBCA | $C_8H_{10}O_2Br_2$ | $[M-H]^-$ | 294.89747 | - | - |
| | | | PNP-D4 | $C_6HD_4NO_3$ | $[M-H]^-$ | 142.04477 | - | - |
| | | | FPBA-13C6 | $^{13}C_6C_7H_9FO_3$ | $[M-H]^-$ | 237.06642 | - | - |
| | | | DCCA-13C2 | $^{13}C_2C_6H_9DCl_2O_2$ | $[M-H]^-$ | 210.01149 | - | - |
| Internal Standards | | | ATZM-13C3 | $^{13}C_3C_{10}H_{22}N_6O_3S$ | $[M+H]^+$ | 346.16475 | - | - |
| | | | 2,4-D-D3 | $C_8H_3Cl_2O_3D_3$ | $[M-H]^-$ | 221.98095 | - | - |
| | | | MMP-D6 | $C_2H_2NO_2PSD_6$ | $[M+H]^+$ | 148.04627 | - | - |
| | | | DIMET-D6 | $C_5H_6NO_3PS_2D_6$ | $[M+H]^+$ | 236.04455 | - | - |
| | | | DBP | $C_8H_{19}O_4P$ | $[M-H]^-$ | 209.09481 | - | - |

## 2.2. Sample Preparation and UHPLC-HRMS.

*2.2. Sample Preparation and UHPLC-HRMS.* A previously developed sample preparation was used [6]. Briefly, in order to hydrolyze the possible glucuronide- or sulfate-conjugated metabolites, 5 mL of urine was mixed with 1 mL of 0.2 M acetate buffer 20 μL of β-glucuronidase aryl sulfatase enzyme and the internal standard solution mix in the analysis of real samples. The samples were maintained at 37°C overnight for hydrolysis.

Metabolites were extracted from the urine samples employing the dispersive solid phase extraction QuEChERS kits. In a 50 mL polypropylene tube, the urine was mixed with 10 mL of acetonitrile, a QuEChERS pouch, and 2 ceramic pieces. After centrifugation, the acetonitrile phase was transferred and evaporated to dryness in a water bath at 37°C under a nitrogen stream. Subsequently, 200 μL of methanol: water (10:90, v/v) containing 0.1% of acetic acid was added and the solution was transferred into a Millipore 0.2 μm Eppendorf and ultra-centrifuged. The final extract was transferred into an injection vial and analyzed in the UHPLC-HRMS system.

Chromatographic separation was performed on an ultra-high performance liquid chromatography (UHPLC) system Accela™ equipped with a Hypersil Gold column (100 mm x 2.1 mm, 1.9 μ) from ThermoFisher Scientific (Bremen, Germany). The chromatographic separation was optimized in a previous study [6]. Briefly, the flow rate used was 400 μL min$^{-1}$ and the injection volume was 10 μL. A binary gradient was used: acetic acid 0.1% (v/v) in water was used as mobile phase A, while acetic acid 0.1% (v/v) in methanol was used as mobile phase B. The analysis started with 95% mobile phase A. After 1 min, this percentage was linearly decreased down to 45% within 5 min. After that, solvent A was decreased quickly to 0% in 0.5 min and maintained for 1.5 min. The total run time was 20 min.

Mass analysis was performed on the Orbitrap mass spectrometer Exactive™ analyzer (Thermo Scientific, Bremen, Germany). The system was equipped with a heat electrospray ionization interface (HESI-II). The ion-source parameters were previously optimized as follows: spray voltage: 3.5 kV (positive mode) and 2.5 kV (negative mode); sheath gas flow rate: 55; auxiliary gas flow rate: 10; skimmer voltage: 23 V; heater temperature: 300°C; capillary temperature: 150°C; capillary voltage: 45 V and tube lens voltage: 120 V. For more details of the HRMS analysis see Roca *et al.* (2014) [6].

## 2.3. Compound Identification Criteria.

*2.3. Compound Identification Criteria.* The criteria for target compound identification were established following the SANTE/11813/2017 guideline [30]: (i) mass accuracy of the molecular ion< 5 ppm; (ii) mass accuracy of the fragment ion < 5 ppm; (iii) isotopic pattern similar to the theoretical isotopic pattern (the relative intensity of the A+1 and/or A+2 isotope peaks in the real sample shall correspond to the theoretical relative intensities). For confirmation we used the reference standard solutions of those compounds available in the market. In this case the confirmation criteria were included: (iv) retention time (t$_R$) similar to that of the reference standard ± 0.1 min.

## 2.4. HRMS Orbitrap Parameters Optimization

*2.4.1. Resolving Power Optimization.* To optimize the Resolving Power (R), the system operating in full-scan mode (50-800 m/z) was tested at the R of 10,000; 25,000; and 50,000 FWHM. 6 blank matrix urine aliquotes spiked with a mixed-standard solution of 24 target pesticide metabolites (see Table 1) (50 ng·mL$^{-1}$) were analyzed.

The Resolving Power was evaluated measuring the peak area (signal intensity) and the mass accuracy (Δm) for the diagnostic and fragment ions of each metabolite. A scheme of the Resolving Power optimization study is detailed in Table SI.1. Resolving Power optimization data were acquired with ESI+/- in separated injections, using HCD fragmentation 20 eV and external mass calibration.

*2.4.2. Fragmentation Optimization.* After the selection of the most suitable R, HCD and CID fragmentations were evaluated. Five spiked urine samples were injected with CID energies of 10, 20, 30, and 40 eV. We previously set the energy for HCD fragmentation to 20 eV. Once the CID energy was optimized, five different methods, in six spiked samples (50 ng·mL$^{-1}$), were studied using or not HCD and CID fragmentations and using ESI+ and ESI- in the same or in different injections. The response was evaluated measuring the peak area of the fragment ions in (i) ESI$^+$ with and without HCD (HCD= 20 eV); (ii) ESI$^-$ with and without HCD (HCD= 20 eV); (iii) ESI$^+$ with and without CID; (iv) ESI$^-$ with and without CID; (v) ESI$^+$ and ESI$^-$ in the same injection with and without CID. Fragmentation optimization data were acquired using the previously optimized R and external mass calibration.

*2.4.3. Internal and External Mass Calibration Study.* With respect to mass calibration, both external and internal mass calibrations were evaluated. External calibration was performed using the mixtures ProteoMass™ LTQ/FT-Hybrid ESI Cal Mix in Pos an Neg Mode (Supelco, Bellefonte, PA, USA). Internal mass calibration was achieved introducing caffeine (M+H$^+$ m/z = 195.08765 Da) in the mobile phase as a lock mass for positive ionization (ESI$^+$).

In total, five spiked aliquotes were analyzed using internal and external mass calibration separately. The analytical response was evaluated measuring peak areas and mass accuracies (Δm) for the diagnostic and fragment ions. All mass calibration study data were acquired using the previously optimized R and fragmentation settings.

Data were processed using the TraceFinder™ 3.1 (Thermo Scientific, Bremen, Germany) and Xcalibur™ 2.2 (Thermo Scientific, Bremen, Germany) software.

# 3. Results and Discussion

*3.1. Resolving Power Optimization.* In order to select the most appropriate R for the determination of pesticide metabolites in urine, the influence of this parameter on the signal and mass accuracy of the 24 compounds was investigated. Tables 2 and 3 show the signal intensity (peak area) and the mass

TABLE 2: Average peak area and coefficient of variation (CV, %) obtained at 3 different Resolving Powers (R) for pesticide metabolites diagnostic and fragment ions (n = 6).

| Metabolite | Peak area | Diagnostic ion | | | Fragment ion | | |
|---|---|---|---|---|---|---|---|
| | | R=10,000 | R=25,000 | R=50,000 | R=10,000 | R=25,000 | R=50,000 |
| CMHC | Average | 908337 | 839333 | 570456 | 12927 | 26726 | 39492 |
| | CV (%) | 1.51 | 2.91 | 7.49 | 24.38 | 21.36 | 52.87 |
| DEAMPY | Average | 994268 | 880944 | 834834 | 63190 | 161187 | 175626 |
| | CV (%) | 17.15 | 28.51 | 10.49 | 22.31 | 16.20 | 40.94 |
| IMPY | Average | 1447089 | 1425080 | 1663104 | Not found | 47097 | 116409 |
| | CV (%) | 0.36 | 7.92 | 13.22 | Not found | 1.23 | 41.28 |
| PNP | Average | 6388647 | 6280026 | 6007343 | 3938342 | 3858484 | 4005830 |
| | CV (%) | 1.70 | 2.06 | 1.92 | 7.36 | 4.40 | 6.67 |
| TCPy | Average | 1090750 | 1148221 | 943686 | - | - | - |
| | CV (%) | 1.73 | 7.04 | 8.08 | - | - | - |
| MNP | Average | 3938892 | 3813784 | 3650660 | 503016 | 474896 | 634023 |
| | CV (%) | 3.10 | 3.85 | 4.18 | 11.40 | 13.27 | 19.46 |
| DMTP | Average | 76340 | 68339 | 88833 | 2644 | 6361 | 9973 |
| | CV (%) | 39.41 | 5.74 | 16.55 | 57.74 | 37.34 | 57.74 |
| DMDTP | Average | 111502 | 321392 | 247711 | 2679 | 16962 | 53675 |
| | CV (%) | 36.39 | 10.13 | 30.71 | 57.74 | 110.51 | 11.56 |
| DEP | Average | 1719737 | 1870997 | 1556112 | 196269 | 272546 | 272666 |
| | CV (%) | 4.09 | 4.33 | 5.83 | 21.00 | 12.49 | 6.12 |
| DETP | Average | 2224008 | 2154624 | 1819644 | 50934 | 141703 | 169339 |
| | CV (%) | 0.08 | 6.97 | 6.56 | 63.42 | 24.47 | 24.00 |
| AP | Average | 58729 | 74307 | 90733 | 157684 | 157729 | 206069 |
| | CV (%) | 8.34 | 33.62 | 17.63 | 7.21 | 4.59 | 13.43 |
| MMP | Average | 69818 | 178190 | 112470 | 19869 | 169131 | 69306 |
| | CV (%) | 7.38 | 2.89 | 4.58 | 25.93 | 3.05 | 7.43 |
| OMET | Average | 45484 | 226016 | 149173 | 13032 | 25505 | 32670 |
| | CV (%) | 69.04 | 23.35 | 17.68 | 35.04 | 22.09 | 32.95 |
| DIMET | Average | 380660 | 399855 | 329466 | 60934 | 83874 | 87121 |
| | CV (%) | 8.48 | 11.44 | 14.44 | 6.51 | 10.55 | 31.27 |
| PBA | Average | 648211 | 571829 | 363693 | 121191 | 138365 | 166373 |
| | CV (%) | 8.82 | 15.34 | 16.11 | 11.30 | 20.44 | 23.68 |
| FPBA | Average | 612579 | 519635 | 405204 | 73743 | 82755 | 112916 |
| | CV (%) | 13.94 | 23.07 | 11.99 | 16.68 | 13.95 | 31.57 |
| cis DCCA | Average | 171464 | 273263 | 194626 | - | - | - |
| | CV (%) | 28.60 | 16.39 | 13.55 | - | - | - |
| trans DCCA | Average | 453810 | 436816 | 314120 | - | - | - |
| | CV (%) | 6.12 | 6.49 | 13.04 | - | - | - |
| DBCA | Average | 6904 | 32503 | 32185 | - | - | - |
| | CV (%) | 31.56 | 40.91 | 16.74 | - | - | - |
| ATZM | Average | 273078 | 1179224 | 756085 | 1314830[a] | 1421843[a] | 1252716[a] |
| | CV (%) | 71.21 | 3.96 | 2.01 | 3.57 | 4.46 | 9.05 |
| ALAM | Average | 67659 | 687712 | 511658 | 831200[a] | 1255816[a] | 1242225[a] |
| | CV (%) | 12.30 | 15.92 | 7.77 | 7.02 | 2.35 | 5.40 |
| METM | Average | 268530 | 644807 | 446473 | 2762 | 5381 | 6657 |
| | CV (%) | 8.80 | 4.93 | 9.49 | 57.74 | 57.74 | 57.74 |
| 2,4-D | Average | 1234998 | 1225331 | 907979 | 1130961 | 1117200 | 1224757 |
| | CV (%) | 6.27 | 4.39 | 5.67 | 3.85 | 8.45 | 3.68 |
| 2,4,5-T | Average | 731224 | 786035 | 477627 | 677796 | 718415 | 777837 |
| | CV (%) | 10.79 | 10.86 | 10.65 | 7.95 | 12.15 | 13.05 |

Acquisition conditions: ESI+/ESI- in separated injections, HCD fragmentation 20 eV, and external mass calibration.
-: no fragment ions monitored.
[a] Irregular peak shape caused by isobaric interferences. The measured areas could be affected by the interferences.

TABLE 3: Average and range of mass accuracies (Δm) (ppm) obtained at 3 different Resolving Powers (R) for pesticide metabolites diagnostic and fragment ions (n=6).

| Metabolite | Δm (ppm) | Diagnostic ion | | | Fragment ion | | |
|---|---|---|---|---|---|---|---|
| | | R=10,000 | R=25,000 | R=50,000 | R=10,000 | R=25,000 | R=50,000 |
| CMHC | Average | 2.27 | 1.38 | 1.24 | 3.11 | 2.52 | 3.36 |
| | Range | 1.60 – 2.75 | 0.07 – 2.32 | 0.93 – 1.65 | 1.61 – 4.16 | 1.10 – 3.81 | 3.33 – 3.40 |
| DEAMPY | Average | 1.09 | 2.68 | 1.30 | 3.19 | 1.70 | 1.44 |
| | Range | 0.05 – 2.58 | 1.65 – 3.66 | 0.73 – 1.90 | 1.70 – 4.53 | 0.69 – 3.37 | 0.49 – 2.38 |
| IMPY | Average | 1.81 | 1.56 | 1.33 | > 5 | 1.61 | 1.99 |
| | Range | 1.25 – 2.14 | 1.36 – 1.76 | 1.26 – 1.36 | > 5 | 1.35 – 1.90 | 1.90 – 2.07 |
| PNP | Average | 2.69 | 1.94 | 1.63 | 3.64 | 2.18 | 1.96 |
| | Range | 2.31 – 3.08 | 0.77 – 2.87 | 1.20 – 1.86 | 2.92 – 4.99 | 1.09 – 3.13 | 1.48 – 2.28 |
| TCPy | Average | 2.23 | 2.01 | 1.83 | - | - | - |
| | Range | 0.28 – 3.01 | 0.42 – 3.12 | 1.05 – 3.38 | - | - | - |
| MNP | Average | 2.59 | 1.32 | 1.59 | 2.36 | 1.18 | 1.89 |
| | Range | 2.56 – 2.66 | 1.05 – 1.56 | 1.15 – 2.06 | 1.70 – 2.93 | 0.88 – 1.70 | 1.62 – 2.11 |
| DMTP | Average | 0.79 | 0.66 | 2.43 | 2.41 | 1.02 | 1.28 |
| | Range | 0.11 – 1.40 | 0.34 – 1.09 | 1.96 – 2.82 | 1.51 – 4.52 | 0.75 – 1.19 | 0.08 – 2.78 |
| DMDTP | Average | 2.47 | 1.49 | 2.14 | 1.96 | 2.67 | 1.31 |
| | Range | 1.26 – 3.21 | 0.87 – 2.52 | 1.65 – 2.43 | 0.25 – 4.03 | 1.67 – 4.26 | 0.49 – 2.10 |
| DEP | Average | 2.66 | 2.02 | 1.84 | 1.88 | 2.05 | 2.35 |
| | Range | 1.43 – 3.26 | 1.16 – 2.84 | 1.44 – 2.14 | 0.89 – 2.81 | 1.03 – 3.11 | 1.98 – 2.71 |
| DETP | Average | 3.46 | 2.02 | 1.96 | 2.26 | 3.13 | 2.10 |
| | Range | 3.28 – 4.99 | 1.27 – 2.63 | 1.30 – 2.29 | 0.61 – 4.22 | 2.30 – 4.22 | 1.31 – 3.04 |
| AP | Average | 1.66 | 1.41 | 1.39 | 1.91 | 2.26 | 1.79 |
| | Range | 0.42 – 4.80 | 0.60 – 2.10 | 0.48 – 2.57 | 0.06 – 4.00 | 1.30 – 3.16 | 1.13 – 2.11 |
| MMP | Average | 2.21 | 1.67 | 1.69 | 3.06 | 1.67 | 3.45 |
| | Range | 0.28 – 4.14 | 1.24 – 2.43 | 0.92 – 2.69 | 2.62 – 3.49 | 1.24 – 2.43 | 3.22 – 3.63 |
| OMET | Average | 0.86 | 1.92 | 2.09 | 1.55 | 3.36 | 1.90 |
| | Range | 0.03 – 1.39 | 1.11 – 2.61 | 1.86 – 2.38 | 0.47 – 2.39 | 2.17 – 4.98 | 1.19 – 2.99 |
| DIMET | Average | 2.79 | 2.55 | 2.13 | 2.38 | 2.34 | 2.67 |
| | Range | 1.18 – 4.62 | 2.18 – 2.92 | 1.14 – 3.10 | 0.38 – 4.06 | 1.10 – 3.90 | 2.20 – 3.11 |
| PBA | Average | 1.79 | 1.33 | 1.12 | 2.25 | 1.55 | 1.77 |
| | Range | 1.25 – 2.11 | 0.18 – 2.85 | 0.68 – 1.97 | 1.65 – 3.25 | 0.28 – 3.53 | 1.18 – 2.26 |
| FPBA | Average | 1.17 | 2.09 | 1.38 | 3.10 | 1.85 | 1.41 |
| | Range | 0.15 – 1.80 | 0.80 – 3.49 | 0.66 – 2.41 | 2.09 – 4.20 | 0.30 – 3.46 | 0.10 – 1.92 |
| cisDCCA | Average | 1.84 | 1.42 | 1.76 | - | - | - |
| | Range | 1.20 – 2.31 | 0.31 – 2.18 | 0.61 – 2.67 | - | - | - |
| transDCCA | Average | 2.37 | 1.82 | 1.41 | - | - | - |
| | Range | 1.05 – 3.47 | 0.23 – 3.04 | 0.31 – 2.45 | - | - | - |
| DBCA | Average | 2.92 | 2.07 | 1.78 | - | - | - |
| | Range | 1.80 – 4.10 | 1.08 – 2.74 | 0.36 – 3.16 | - | - | - |
| ATZM | Average | 3.61 | 2.29 | 2.98 | 1.18 | 1.84 | 1.85 |
| | Range | 2.29 – 4.97 | 1.43 – 2.87 | 2.17 – 4.12 | 0.83 – 1.63 | 1.42 – 2.32 | 1.56 – 2.27 |
| ALAM | Average | 3.41 | 2.57 | 3.29 | 2.30 | 2.14 | 1.96 |
| | Range | 1.24 – 4.76 | 1.69 – 2.85 | 2.85 – 4.76 | 1.38 – 3.25 | 1.61 – 3.38 | 1.61 – 2.43 |
| METM | Average | 2.37 | 2.68 | 2.77 | > 5 | 1.52 | 2.13 |
| | Range | 0.90 – 4.38 | 1.70 – 3.87 | 1.19 – 4.31 | 1.09 – >5 | 0.42 – 2.35 | 1.82 – 3.13 |
| 2,4-D | Average | 2.65 | 1.92 | 1.72 | 3.21 | 2.19 | 1.89 |
| | Range | 1.98 – 3.62 | 0.97 – 2.56 | 0.74 – 2.77 | 2.82 – 3.87 | 1.08 – 3.00 | 1.76 – 2.14 |
| 2,4,5-T | Average | 1.65 | 1.30 | 2.28 | 2.89 | 2.15 | 1.71 |
| | Range | 0.46 – 2.26 | 0.22 – 1.85 | 1.30 – 4.14 | 2.16 – 3.65 | 1.42 – 2.78 | 1.29 – 2.23 |

Acquisition conditions: ESI+/ESI- in separated injections, HCD fragmentation 20 eV, and external mass calibration.
-: no fragment ions monitored.

TABLE 4: Number of ions (diagnostic and fragment ions) into the six mass accuracy ranges considered at 10,000, 25,000, and 50,000 FWHM.

| Resolving Power | Δm (ppm) | | | | | |
|---|---|---|---|---|---|---|
| | ≤ 1 | ]1 – 2] | ]2 – 3] | ]3 – 4] | ]4 – 5] | > 5 |
| 10,000 | 2 | 12 | 19 | 9 | 0 | 2 |
| 25,000 | 1 | 22 | 19 | 2 | 0 | 0 |
| 50,000 | 0 | 30 | 11 | 3 | 0 | 0 |

Acquisition conditions: ESI+/ESI- in separated injections, HCD fragmentation 20 eV, and external mass calibration.

accuracy (Δm), respectively, of the diagnostic and fragment ions.

Regarding diagnostic ions, some specific metabolites such as DEAMPY, IMPY, PNP, TCPy, MNP, DEP, DETP, or DIMET presented similar areas at the three R checked (Table 2). However, R = 25,000 FWHM provided the largest areas for DMDTP, MMP, OMET, cis DCCA, DBCA, ATZM, ALAM, METM, and 2,4,5-T. For the remaining 7 compounds a R= 25,000 FWHM presented areas close to that provided by the best R for each compound (10,000 FWHM or 50,000 FWHM).

For fragments, 17 out of 20 compounds presented higher intensities with R = 50,000 FWHM (Table 2). In some cases, as for METM, fragment ions areas are very low in comparison with the diagnostic ion response. It is relatively frequent that AIF produces fragment ions with low abundances, partly because the optimal fragmentation energy is not equal for all diagnostic ions. It could affect the analysis of real samples, because some diagnostic ions may not be confirmed because their fragment ions signal is below the noise. To sum up, R = 25,000 FWHM seems more suitable for diagnostic ions and 50,000 FWHM for fragment ions. However, taking into account the total ions (diagnostic plus fragments) and the variability of the response, a R = 25,000 FWHM was selected because it presented more number of ions with high area (see Figure SI.1). We have not found a clear explanation why some ions present higher responses when acquired at a R (e.g., 25,000 FWHM) while other ions present higher responses when acquired at another R (e.g., 50,000 FWHM).

In addition, the R of 25,000 FWHM presented more data points (scans) per peak than R = 50,000 FWHM because in an Orbitrap analyser the scan speed decrease when R increase (i.e., 2 Hz at R = 50,000 FWHM; 4 Hz at R = 25,000 FWHM). The number of data points is important for peak shape and quantification.

Table 3 shows the average and range of mass accuracy (Δm, ppm) of pesticide metabolites diagnostic and fragment ions obtained at the three R tested. Table 4 summarizes the results showing the number of ions in each of the six ranges of mass accuracy considered. As can be observed (Table 4), good mass accuracies (<3 ppm) in most of the ions were obtained applying R of 25,000 and 50,000 FWHM.

Taking into account these results, a R = 25,000 FWHM was chosen because (i) it gave the highest signal (peak area) for more analytes; (ii) it presented good mass accuracy; and (iii) the scan time gave a suitable number of data points. An added advantage of using this intermediate R is that the instrument presents a sufficient speed to be able to use the detection of positive (ESI+) and negative (ESI−) ions
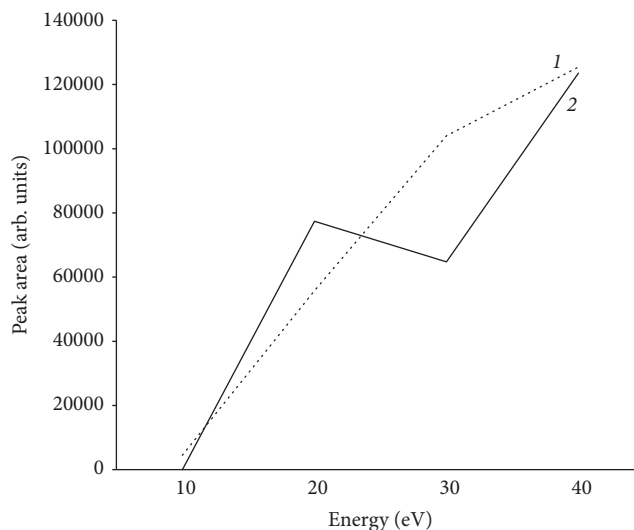


FIGURE 1: CID fragmentation of PBA (m/z = 169.06589). Variation of the fragment ion response (peak area) with the applied energy (eV). (1) Applying only ESI+; (2) using the polarity switching (ESI+, ESI− in the same injection). Acquisition conditions: R= 25,000 FWHM and external mass calibration.

in the same injection, increasing the speed of analysis and the throughput of the method. Martínez-Dominguez *et al.* (2016) also applied R = 25,000 FWHM for the analysis of organic contaminants in food using a UHPLC-Orbitrap detector [12]. QTOF systems also employed similar Resolving Powers (18,000-22,500 FWHM) for the analysis of pesticides, organophosphate flame retardants, and chemical agents in urine and drinking water [15, 16, 31]. However, in Orbitrap analyzer a higher Resolving Power (50,000 FWHM) was used for the analysis of pesticides and drugs in urine, food, and feed [6, 11, 27] and for the analysis of mycotoxins in food (70,000 FWHM) [14]. In contrast, a lower Resolving Power was employed in other studies ranging from 7,000 to 17,500 FWHM in biological and food samples [7, 12, 18]. Kaufmann *et al.* (2010) compared liquid chromatography selectivity in LC-MS/MS and LC-HRMS, applying different Resolving Powers (10,000-100,000 FWHM). They indicated that a Resolving Power of 50,000 FWHM was the most suitable for the analysis of veterinary drugs in food using LC-Orbitrap detector [22].

*3.2. Fragmentation Optimization.* Regarding the optimization of the CID energy, the highest fragment areas were obtained using a fragmentation energy of 40 eV. As an
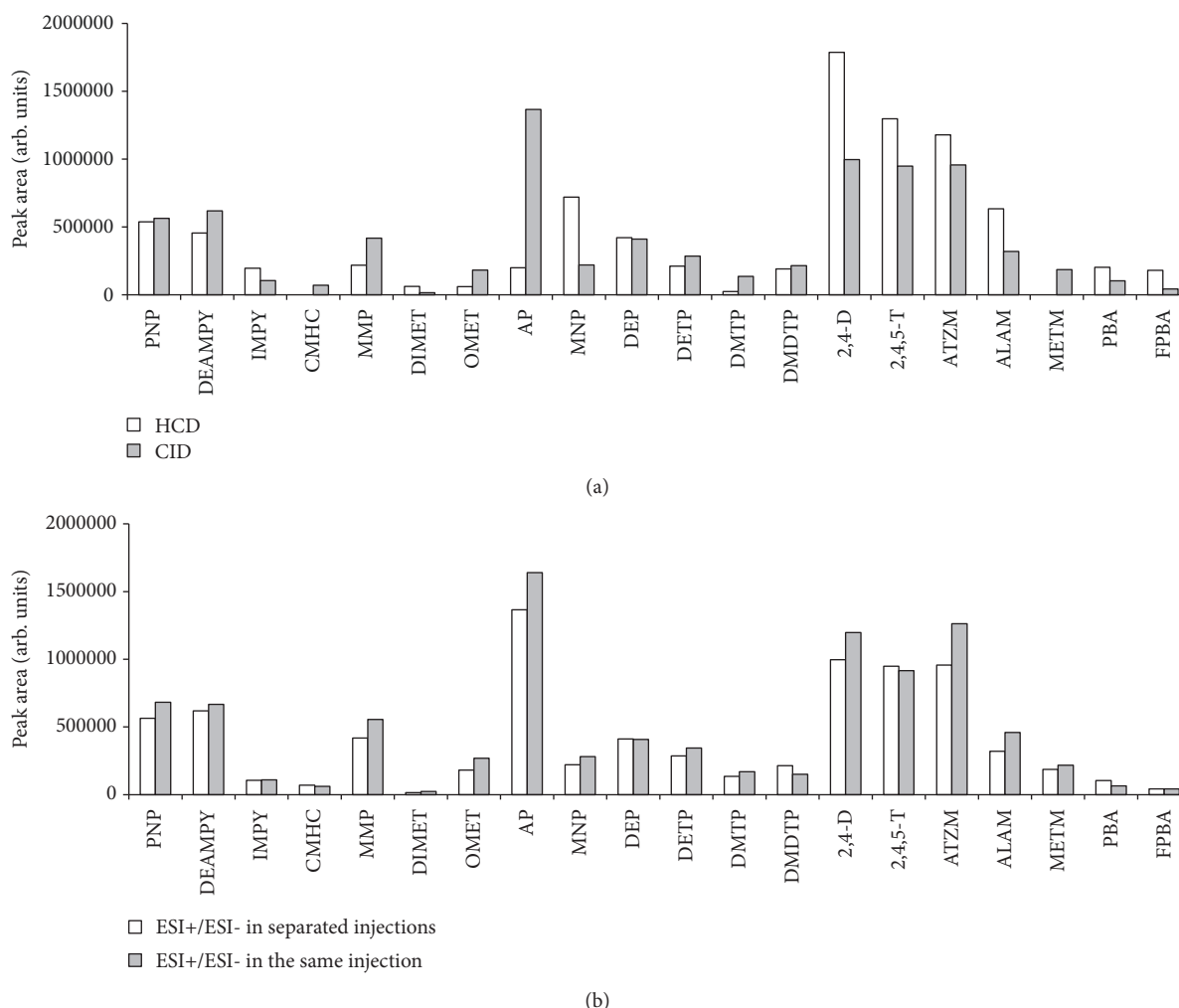
FIGURE 2: (a) Comparison between responses (peak area) of fragments obtained using CID (40 eV) and HCD (20 eV). (b) Comparison between CID fragmentation (40 eV) using the polarity switching function (ESI$^+$ and ESI$^-$) in the same injection and in two separate injections. Acquisition conditions: R= 25,000 FWHM and external mass calibration.

example, Figure 1 shows the fragment ion areas obtained using CID at different energies for PBA.

Comparing the results obtained using CID and HCD, Figure 2(a) shows similar areas for fragment ions obtained with CID and HCD modes. However, some compounds such as CMHC and METM were only fragmented applying CID. Consequently, CID energy (40 eV) was selected for all compounds. Similar results were obtained using ESI$^+$ and ESI$^-$ in the same injection than in two separate injections (see Figure 2(b)). Consequently, a method with ionization mode in ESI$^+$ and ESI$^-$ in the same injection and CID fragmentation was selected. Strano-Rossi *et al.* (2015) also selected CID energy (40 eV) in LC-Orbitrap for the determination of stimulants and drugs in food supplements [27]. Tak *et al.* (2014) employed higher CID energy (150 eV) in an LC-QTOF detector for the determination of chemical warfare agents in drinking water [31]. However, HCD is the most widely fragmentation mode used in HRMS Orbitrap detectors. HCD energies from 20 to 30 eV have been

applied to fragment compounds such as pesticides, biotoxins, mycotoxins, veterinary drugs, and other toxins in biological, food, and feed samples [6, 11, 12, 18, 20]. Optimization of the mode of fragmentation in Orbitrap detectors is also carried out in other fields as proteomics. Jedrychowski *et al.* (2011) have evaluated CID/HCD fragmentation for murine phosphoproteomics [28].

*3.3. Internal and External Mass Calibration Study.* In general, the use of lock mass (internal calibration) improves mass accuracy. In addition, in the present method some fragments are below 138.06619 Da, which is the low mass in the ESI$^+$ external calibration solution; consequently a continuous correction of the acquired masses (internal calibration) could avoid an excessive mass drift.

In order to check whether this general rule is applicable to this particular application, we studied the influence of the calibration mode on the mass accuracy of eight substances analyzed in ESI$^+$ mode, using caffeine as internal

TABLE 5: Average mass accuracies (Δm) (ppm) and standard deviations (Std dev) using internal and external calibration for diagnostic and fragment ions in ESI positive mode (n=5).

| Metabolite | Diagnostic ion | | | | Fragment ion | | | |
|---|---|---|---|---|---|---|---|---|
| | Internal calibration | | External calibration | | Internal calibration | | External calibration | |
| | Δm (ppm) | Std dev | Δm (ppm) | Std dev | Δm (ppm) | Std dev | Δm (ppm) | Std dev |
| DEAMPY | 0.21 | 0.13 | 0.47 | 0.26 | 0.17 | 0.08 | 1.15 | 0.19 |
| IMPY | 0.08 | 0.06 | 0.62 | 0.26 | 3.55 | 0.08 | 2.64 | 0.23 |
| DIMET | 0.27 | 0.07 | 0.76 | 0.29 | 0.44 | 0.05 | 0.50 | 0.12 |
| OMET | 0.14 | 0.10 | 0.53 | 0.39 | 0.12 | 0.11 | 0.90 | 0.33 |
| AP | 0.12 | 0.10 | 0.41 | 0.21 | 0.15 | 0.08 | 0.99 | 0.27 |
| ATZM | 0.49 | 0.15 | 0.99 | 0.41 | 0.19 | 0.04 | 0.91 | 0.36 |
| ALAM | 0.24 | 0.27 | 1.14 | 0.55 | 0.17 | 0.14 | 0.59 | 0.17 |
| METM | 1.52 | 1.38 | 2.23 | 1.07 | 0.77 | 0.45 | 1.96 | 0.39 |

Acquisition conditions: ESI+/ESI- in the same injection, R = 25,000 FWHM, and CID fragmentation 40 eV.

standard. Table 5 shows the mass accuracies (Δm, ppm) for the diagnostic and fragment ions obtained with internal and external mass calibration. As it can be observed, a better Δm was obtained when working with internal mass calibration. Hence, the use of caffeine as lock mass was selected.

In general, external mass calibration is used in the literature for HRMS Orbitrap detector and internal mass calibration for HRMS QTOF detectors. External mass calibration of Orbitrap in urine, plasma, food, and feed has been widely performed for pesticides, parabens, veterinary drugs, biotoxins, mycotoxins, and other substances [6, 8, 11, 12, 18, 20]. However, internal mass calibration has also been used, as in the method described by Strano-Rossi *et al.* (2015), for the analysis of various stimulants in food supplements. In this case the authors used diisooctyl phthalate ionic species, m/z 391.2843 Da, in order to compensate any possible mass axis drifts, obtaining Δm < 1 ppm for all ionic species [27]. In contrast, QTOF detectors normally calibrate with negative internal mass calibration (leucine enkephalin) [15, 16, 19] or positive internal mass calibration (purine and hexakis-(1H,1H,3H-tetrafluoropentoxy)phosphazene) [31].

## 4. Conclusions

Mass spectrometry parameters such as Resolving Power, fragmentation mode, and type of mass calibration have been optimized for the analysis of 24 pesticide metabolites in urine. A Resolving Power of 25,000 FWHM, internal calibration, and CID fragmentation were selected as the best options to improve the signal intensity and the mass accuracy of diagnostic and fragment ions. This Resolving Power provides enough resolution to avoid isotopic interferences and allows the use of the polarity switching function (ESI$^+$ and ESI$^-$ in the same injection), hence reducing the analysis time.

The optimized HRMS parameters allow the determination of pesticide metabolites in urine samples; however a further validation of the method is required to determine the LOD and other performance parameters.

## 5. Study Limitation

We have not tested CID energies higher than 40 eV.

## Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

## Acknowledgments

## Supplementary Materials

Table SI.1: scheme of the Resolving Power optimization study. Figure SI.1: number of ions with high ([100-80]%) and low (<80%) peak areas at R=10,000, R=25,000, and R=50,000 (FWHM), for total, diagnostic, and fragment ions. *(Supplementary Materials)*

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