Advantages of MS/MS/MS (MRM3) vs classic MRM quantification for complex environmental matrices: Analysis of beta-lactams in WWTP sludge

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Abstract

When dealing with complex matrices such as wastewater treatment plant (WWTP) sludge or animal manure, usual MRM quantification may lack enough sensitivity or accuracy due to the presence of numerous interfering compounds co-extracted from the matrix. To circumvent the sensitivity and specificity loss, the method development can be focused on sample extraction, purification or/and optimization of the detection. In this study, we propose an enhancement of a method for the analysis of five beta-lactams (Amoxicillin, Ampicillin, Cefapirin, Ceftiofur, and Cloxacillin) in WWTP, with the use of a hybrid triple quadrupole-Linear Ion Trap (LIT) spectrometer, enabling triple stage MS acquisition, namely MRM^3, in place of the usual MS/MS detection. The adaptation of various parameters such as the secondary fragmentation energy, excitation, and accumulation times of the secondly generated ion are described. The method was then validated and enabled quantification limits between 0.8 and 14.7 $ng.g^{-1}$ associated with accurate quantification (between 98% and 113%). This method is the first to report the use of MRM^3 acquisition in an environmental matrix as complex as sludge.

Keywords: MS/MS/MS, solid matrix, quantification, antibiotics, environment

1. *Introduction*

For decades, LC-MS/MS with acquisition in Multiple Reaction Monitoring (MRM) has been widely used for the targeted quantification of small exogenous molecules (typically < 600 Da) such as pesticides or antibiotics in all sorts of matrices either biological (urine, blood, plasma…), food related (milk, honey, fruits, and vegetables…) or environmental (wastewaters, sediments…). Through parent ion selection, fragmentation, and 1st generation fragment selection, the MRM mode showed increased sensitivity and specificity over Single Ion Monitoring (SIM)[1] and a wider linear dynamic range. However, in highly complex matrices such as wastewater sludge, the removal of interfering compounds might not be complete, leading to higher detection and quantification limits. Nowadays, some triple quadrupoles mass spectrometers are equipped with a linear ion trap (LIT) in place of the third quadrupole. This development enables filtering and trapping of an ion previously generated in the collision cell. This ion can then be further fragmented and accumulated in the LIT before detection, enhancing the specificity and sensitivity. With this dual stage fragmentation, this MS-based strategy is called MRM cubed (MRM^3).

MRM^3 has been mainly employed to analyse biological matrices, for proteomic applications. For instance, MRM^3 was used by Lemoine *et al.* [2–6] for proteins and biomarkers determination and quantification in biofluids. Quantification limits enhancements and highly specific detection of the various analytes were reported to the point that sample preparation may be eased or even removed. Specific detection of pork and horse protein markers [7,8] was reported, with diminution of the quantification limits by a factor between 2 and 100 compared to MS/MS. Onorato *et al.* [9] used MRM^3 for quantification of a glycemic biomarker in patient urine and reported accurate measurement of the marker within 8% of the theoretical value. Some methods using MRM^3 determination were also described for quantification of small molecules in plasma [10–15] or in foodstuff for which this mode was employed for allergens quantification [16–18].

Considering environmental matrices, the use of MRM^3 is rare. Sordet *et al.* developed the MRM^3 acquisition for determination of X-ray contrast agents in wastewaters [19] or different emerging micro pollutants in a crustacean [20]. In this last study, the gain in sensitivity allowed to detect both inter-samples variations and small uptake variations across consecutive days in the micro invertebrate. Finally, in another study related to an environmental species, Smith *et*

al. [21] used an ion-trap mass spectrometer for the determination of 6 drugs in fish species with MRM^3, to improve the specificity as the major MRM transition corresponded to a water loss.

The analysis of traces of contaminants in sludge is a real challenge. Indeed, this matrix is extremely dense and complex, containing a large number of organic molecules with very diverse physicochemical properties [22–24]. From an environmental point of view, the monitoring of their contamination, including micropollutants,is important because in some countries, notably France, sludge is spread on soils in order to enrich them, which represents a risk of contamination of groundwater. In particular, sludge can contain antibiotics, the presence of which in the soil can lead to antibiotic resistance [25,26]. Although LC-MS/MS has been used to quantify micropollutants in sludge, the quantification limits are sometimes not compatible with (ultra)traces analysis[27]. To the best of our knowledge, MRM^3 has never been considered before for traces analysis in wastewater sludge, or similar highly complex matrices.

In the current study, we developed an LC-MRM^3 method for the quantification of five beta-lactams (Amoxicillin, Ampicillin, Cefapirin, Ceftiofur, and Cloxacillin) extracted from wastewater treatment plant sludge. Beta-lactams are antibiotics widely used in the management and treatment of bacterial infections. These antibiotics are to this day widely used in both veterinary (15% in 2018 [28]) and in human medicine (13% in average on the last 20 years [29]).They are known to be excreted without metabolization from organisms [30], partly explaining their likeliness to be detected in wastewater sludges [31,32].They are also knownto be easily degraded in the environment resulting in the need of quantification at trace level. The aim of this work was to develop MRM^3 acquisition to enable sensitive and accurate quantification of the beta-lactams selected in a complex matrix such as WWTP sludge and to compare performances in MRM and MRM^3.

2. *Experimental*

2.1. *Chemicals*

Ampicillin (AMP) trihydrate, Amoxicillin (AMX) trihydrate, and Cloxacillin (CLX) were bought from TCI Europe (Zwijndrecht, Belgium). Ceftiofur (CEF) and Cefapirin (CFP) were supplied by Sigma-Aldrich (Saint Quentin Fallavier, France). CEF-d3, AMP-d5 and CFP-d4 were bought from TRC (Toronto, Canada). All standards purities were superior to 97%. The

structure of each beta-lactam is reported in Fig.S1. Solutions (1 $mg.mL^{-1}$) of each beta-lactam were prepared by dissolving about 10 mg powder, accurately weighted in 10 mL of acetonitrile (ACN) in glass Wheaton and were stored at -18°C for 3 months. Each standard purity was taken in account for accurate concentration determination. Quantification solutions of each analyte (500 $ng.mL^{-1}$) were prepared by diluting each stock solutions in water/ACN (1/1, v/v). Water (LC-MS grade) was obtained from Fisher Scientific (Illkirch, France), ACN (LC-MS grade) from Honeywell (Seelze, Germany) and formic acid (UPLC-MS grade) from Biosolve (Dieuze, France).

2.2. *Sample collection and preparation*

The sample collection and preparation were described in detail by Guironnet *et al.* [33]. Briefly, 500 mg of freeze-dried sludge was mixed with 2 g of EDTA-treated sand before dispersive-QuEChERS extraction was performed using the citrate buffer with 5 mL of 0.1 M EDTA and 10 mL of ACN. Additional SPE pass-through clean-up completed the sample preparation.

2.3. *LC-MS/MS/MS method*

The system used was an Agilent (Massy, France) 1290 Infinity Series equipped with a quaternary pump. The column was a Kinetex F5, 100×2.1mm, 1.7 µm from Phenomenex (Le Pecq, France). Chromatographic conditions were as followed: a binary mobile phase was used with a flowrate set to 300 $\mu L.min^{-1}$ for a run time of 13 min, with the column maintained at 50°C. Mobile phase A was an aqueous solution of 0.1% formic acid, and B was ACN with 0.1% formic acid. The separation was performed with the following gradient: from 0 to 10 min, a linear gradient from 3% to 100%B, followed by 100%B during 2 min. An equilibration time of 4 min was realised before each injection, leading to a total run time of 16 min. The sample injection volume was 40 µL.

A 5500 QTrap from Sciex® (Les Ulis, France) was used in MS/MS/MS (MRM^3) mode with positive electrospray ionization. Source parameters are detailed in Table S1. MS/MS/MS detection was performed by infusion of individual standard solutions at 100 $ng.mL^{-1}$ in 50/50 H_2O/ACN with 0.1% formic acid, via syringe pump at a flow of 10 $\mu L.min^{-1}$ and are presented in Table S2. Three key parameters needed to be tuned: the fragmentation energy used to

generate the second-generation ion (AF2), the duration on which this energy is applied (excitation time) and the accumulation time of the previously generated ion (LIT time). Each parameter was optimised sequentially and manually by acquiring the MRM^3 spectras averaged over 1 minute

The final acquisition in MRM^3 mode was divided in four periods: from 0 to 2.5 min for AMX; between 2.5 min and 4.25 min for AMP, CFP, and their associated deuterated internal standards; from 4.25 to 6 min for CEF and CEF-d3; and after 6 min for CLX.

2.4. ***Method validation***

All the parameters selected for method validation described in this part were determined and monitored in accordance with both the AOAC [34] and the ICH guidelines [35] for environmental validation. The validation procedure was realised on five days. Limits of quantification (LOQ) for each of the selected analytes were determined by extracting a matrix-blank each day and injecting it ten times. Signal intensities of each blank were recorded and the associated average with standard deviation computed. The intensity corresponding to the LOQ was determined to be the sum of average blank intensity and ten times the standard deviation measured. The method linearity for each molecule was determined by injection of six matrix-matched mixtures from 10 $ng.g^{-1}$ to 200 $ng.g^{-1}$. Calibration curves and samples were spiked with a mixture of internal standards (IS) at 100 $ng.g^{-1}$. Internal calibration was done using the relation between A_{STD}/A_{IS} and C_{STD}/C_{IS}, with A_{STD} being the area of the standard, A_{IS} the area of the IS, C_{STD} and C_{IS} being the concentration of the standard and of the IS respectively. Intraday repeatability was based on 4 replicates; intermediate precision was evaluated on five days. Each day, a matrix-matched calibration curve was freshly extracted and injected, followed by three samples spiked at three concentration levels, namely 20 $ng.g^{-1}$ (LOW), 100 $ng.g^{-1}$ (MID) and 200 $ng.g^{-1}$ (HIGH) also freshly extracted. For each analyte and each level, the concentration was computed with the calibration curve and the accuracy calculated with the mean of the replicates versus the nominal concentration. Intraday repeatability was determined for each level by calculating the relative standard deviation (RSD) of replicates and interday precision was determined by calculating the RSD on five days measurements.

3. *Results and discussion*

3.1. *Optimisation of MRM³ parameters*

All discussions in this section use CEF as an example. Similar process was realised for all other molecules and their internal standard. The results of the development steps are summarized in Table S2.

Firstly, each compound was infused at 100 ng.mL^{-1} in 50:50 water: ACN. As MRM^3 is the fragmentation of a 1st generation fragment ion, the first step consists in the development of the MRM parameters, which was described in Guironnet *et al.* [33]. In case of CEF, the selected MRM transition was *m/z*=524 (P) to *m/z*=241 (F). From this (F) ion, the parameters AF2 energy, excitation time, LIT time and Q0 trapping were sequentially evaluated.

3.1.1. *AF2 energy*

The AF2 value describes the tension applied in the linear trap to fragment the ion generated by the MRM selected method. Increasing fragmentation energy revealed three predominant second-generation ions F': *m/z*=166 (Frag1); *m/z*=197 (Frag2) and *m/z*=209 (Frag3), as seen in Figure 2.

With only 0.05 V applied, the (F) ion quantity was reduced to 28% of the overall ion presence, while Frag2 was major at 47% of the total (Figure 1). When increasing the voltage to 0.1 V, it was noted that the (F) ion was fully converted to Frag 1/2/3, with the ratio 50/37/13, switching the major ion to Frag1. Increase beyond 0.1V revealed slight increase of Frag1, between 1% and 3% for each 0.05 V added. Frag2 was decreased with the same rate, suggesting a conversion from Frag2 to Frag1 rather than an increase in the transformation from (F) to Frag1. Frag3 was rather stable representing in average 12% of the fragmentation products. The detailed evolution of the relative ratio of the fragments is presented in Figure 1 and potential chemical structures are proposed in Figure 2. To obtain accurate determination and no quantification overlap with the competing fragmentation reactions, the choice to follow and quantify only one fragment was made. Here, for the fragmentation of CEF, Frag1 was selected, with a fragmentation energy of 0.1 V.

3.1.2. *Excitation time*

After the selection of the fragmentation energy applied, the next parameter to be fixed is the application duration (in millisecond). With AF2 energy only applied for 1 ms, no fragmentation

was noted (Figure 3). When fragmentation was applied for 5 ms, about a third of the 1st generation fragment was further fragmented to Frag1. Increasing the application length further to 10 ms, Frag1 ion became the major ion detected, with 98% of the overall total. With an excitation time of 25 ms or 50 ms, 1st generation fragment was fully fragmented to *m/z*=166.. To acquire more data points throughout the chromatographic peak, the duty cycle time should be maintained to its lowest possible value. As both 25 ms and 50 ms excitation time resulted in 100% fragmentation, an excitation time of 25 ms was selected.

3.1.3. *LIT time*

For increasing the sensitivity, accumulation of the newly formed 2nd generation fragment is enabled in the linear trap. Two modes are available for accumulation: dynamic or fixed. When dynamic LIT time is employed, accumulation time depends on the ion flux entering in the linear trap. While dynamic accumulation seems to be attractive, the cycle time will shift from one analysis to another depending on the analyte concentration in the extract. The overall reproducibility and accuracy would then be reduced. To ensure the same cycle time throughout the analyses, a fixed LIT time is recommended in MRM^3. With the 5500 QTrap, the LIT time can vary from 1 ms to 250 ms. When infusing CEF with increasing LIT time, an increase of Frag1 signal intensity is noted up to a threshold, as illustrated in Figure 4. For instance, when increasing the LIT time from 1 to 5 ms, the intensity of Frag1 was massively increased by almost 300%. A signal increase of about 68% was observed when the LIT time was increased from 5 to 10 ms, and from 10 to 25 ms.. With each increase of LIT time up to 100 ms, the signal intensity was improved by a factor between 10 and 70%. Increase of the fill time beyond 100 ms reported only increases below 5% with each step, whereas the accumulation duration was effectively increased by a factor between 25% and 50%.

Albeit a signal improvement was noted when increasing the LIT time, the total cycle time also increased, thus reducing the number of points per chromatographic peak. It is now commonly accepted that good peaks should include between 15 and 25 points. A compromise between signal improvement and loss of peak points then needs to be achieved. For CEF, the optimal LIT time was 100 ms. In previous studies, Sordet *et al.* [19,20] and Jaffuel *et al.* [4] presented the LIT time as a non-compound related parameter to be fixed identical for all compounds. In our study, we adapted the LIT time for each compound, and we noted that the optimum value was different, ranging from 100 to 250 ms (Table S2). In their evaluation of MRM^3 for the

quantification of 6-sulfatoxymelatonin in urine, Lopukhov *et al.* [36] also noticed a compound related effect for the LIT time. With higher LIT time, degradation of their analyte occured due to co-accumulation of other matrix substances, and the authors state that an extended study with various LIT time on several substances is required to truly understand the occurring process. This study confirms our claim that LIT time is a parameter worthy of adaptation for each and every substances acquired with MRM^3, especially when dealing with complex matrices. Using fixed LIT time also enables the activation of the Q0 trapping parameter to further enhance the detection.

3.1.4. *Q0 trapping*

Q0 trapping enables further ion focalisation in the ion guide preceding the first quadrupole. By adding a focalisation layer, the ion transfer from the source to the analyser is magnified, thus tending to lower quantification limit. For CEF, adding the focalisation step resulted in a 10% signal gain in infusion. The enhancement of each compound after the Q0 trapping is demonstrated in Figure 5, from 10% for CEF up to 185% increase for CFP.

3.1.5. *Overall enhancement*

When comparing the signal obtained right after the AF2 fragmentation with the optimised signal, major enhancement factors were observed. Regarding CEF, the intensity was multiplied by 36. For the other molecules analysed in this study and presented in Table 1, the lowest enhancement was for AMX with a factor of 4 and the highest was for CFP with a factor of 255.

3.2. *Validation*

Validation was realised on 5 consecutive days on spiked sludge extracts. The results are compiled in Table 2.

3.2.1. *Quantification limits*

Validation of an analytical method based on MRM^3 acquisition can be complex, especially for quantification limits determination. As the second fragmentation adds specificity to the determination, background noise levels are significantly dropped. The assessment of the limits of quantification based on the determination of the concentration corresponding to a signal-to-noise ratio of 10 cannot be performed accurately. Limits of quantification were so determined based on the standard deviation and the averaged intensity of blank sample extracts. With this

method, quantification limits were determined to be between 0.8 $ng.g^{-1}$ for CEF and 14.7 $ng.g^{-1}$ for CLX. For AMX and CLX, the quantification limits were higher than 10 $ng.g^{-1}$ due to the low overall response factor in MS of those two analytes, an effect already noted in the MRM mode. In the presented method, we decided to perform the MRM^3 acquisition with only one second generation ion. To potentially further reduce the achievable quantification limits and detect compounds in (ultra-)traces amounts, it may be useful to cumulate different secondary generation ions to reconstruct the MRM^3 chromatogram. For instance, the CEF MRM fragment has generated three distinct ions at *m/z*=166, *m/z*=197 and *m/z*=209. We only acquired the signal of *m/z*=166, and addition of the others intensity may result in an increase of signal/noise ratio and then a decrease of the quantification limits. However, multiple simultaneous transitions acquisition would also result in a cycle time increase, lowering the number of points of each peak. Different combinations then need to be tested to achieve an optimum.

3.2.2. *Intraday and intermediate precision*

Intraday repeatability was evaluated at three levels: 20 $ng.g^{-1}$; 100 $ng.g^{-1}$ and 200 $ng.g^{-1}$. At the lowest level, the maximum %RSD observed was for CLX with 24%. At the two other levels, intraday variations diminished to a maximum of 17% and 11%, respectively. For interday precision, quantification was realised between 98% and 113% for the 20 $ng.g^{-1}$ level, with the maximum quantification error for AMX. For mid-level and high-level, quantification accuracy is further reduced to ±7% and ±2% for all molecules. Quantification with MRM^3 mode is therefore highly accurate in complex matrices such as wastewater sludge.

3.3. *Comparison with MRM*

As shown in section 3.1, the use of MRM^3 highly increased the signal compared to the widely used MRM. This signal increase resulted in both sensitivity and specificity enhancement, thus allowing the lowering of the quantification limits.

3.3.1. *Sensitivity*

As shown in section 3.1.3, significant increase in signal during infusion was observed. Gains were also compared after coupling with liquid chromatography (chromatograms in Figure S2). At a minimum, the signal for AMP was increased by a factor 12, whereas the signal of CEF was multiplied by 123 (Table 3) when the compounds were in solvent. Regarding matrix

extracts, gains were also noted but were lower than in solvent. For instance, in CEF calibration curves (Figure 6), the slope of the MRM^3 calibration was multiplied by an average factor of 95 compared to MRM slope. This may be explained by the matrix input, from which the extracted interferents create negative matrix effects reducing the positive effect obtained with the MRM^3 acquisition. Lower overall gains were observed for the subgroup of penicillins (AMP, AMX and CLX), maybe due to the m/z of 114 of the final fragment, which is at the low end of the mass range. It is also worth noticing that both in MRM and MRM^3, they exhibit a lower response factor. These lower factors may also be explained by their lower structural stability, the 4 atoms ring being better stabilized in cephalosporins with the associated six atoms ring and a double bond, whereas, in penicillins, it is only stabilized with a 5 atoms ring.

To conclude, even if the gain in intensity is limited in matrix, the increase in slope allows better detection of concentration variations in-between samples. Ultimately, better quantification accuracy will then be achieved.

3.3.2. ***Specificity***

With the addition of a second fragmentation, the specificity of the detection is enhanced in MRM^3 acquisition. Extracted matrix interferents that co-eluted with the analytes increased the noise in the MRM acquisition, as shown with the signal of the internal standard for ampicillin in Figure 7a. In this chromatogram, we can observe a shoulder at the end of the peak, and small peaks eluting right after the internal standard. In MRM^3 (Figure 7b), the removal of both the shoulder and a lowering of the noise after the eluting compound were observed. The noise diminution may also be partially explained with the signal increase brought by the MRM^3 acquisition. The use of MRM^3 can bring better quantification accuracy by being more specific compared to MRM.

3.3.3. ***LOQ enhancement***

Combining sensitivity and specificity enhancements led to lowering the quantification limits achievable in WWTP sludge. When comparing the reached quantification limits between MRM and MRM^3 acquisition, two tendencies emerged. For AMX, CEF and CFP, the quantification limits were reduced by a factor of 2. In this case, the use of developed MRM^3 method led to a sufficient increase of signal and lowering of the noise to allow a gain to detect smaller analytes concentrations. In contrast, LOQ of AMP and CLX were increased by a factor 2. It is worth noting that both their second-generation transitions are the same, following the fragmentation of the ion *m/z*=160 to *m/z*=114, whose hypothetical structures are presented in Figure S3

(Fragmentation of CPF and CEF are reported in Figure S4 and S5). This transition was the one with the smallest mass difference of 46 Da. This additional loss of sensitivity and higher limits of quantification can be explained by an increase of the background noise of these transitions, due to relatively low fragment ion masses (less than 200 Da) and a neutral loss of only 46. This phenomenon may be further heightened by the accumulation time in the trap.

4. *Conclusions*

This study was oriented towards the analysis in WWTP sludge of beta-lactams, widely used antibiotics known to be easily degraded in environmental samples and thus requiring highly selective and sensitive analysis. We developed a step-by-step method to evaluate the enhancement brought by the MRM^3, compared to the classical MRM. We showed a gain in sensitivity for all the analytes, with higher slopes of each calibration curve, allowing better detection of concentration variations in-between samples. The use of MRM^3 also enabled better detection specificity with the removal of interfering matrix compounds.

During the development, we noted different behaviours depending on the analyte. Penicillins showed lower enhancements than cephalosporins, with lower MS-response factors. This optimisation led to attain quantification limits between 0.8 $ng.g^{-1}$ and 14.7 $ng.g^{-1}$, which were, for most of them, below the quantification limits achieved by the LC-MRM method previously developed. For AMP and CLX, higher LOQs were achieved in MRM^3, due to their fragmentation pattern located in the low mass range, revealing the need for specific parameters selection to really lower the LOQs achievable. At the 3 different validation levels, quantification was accurately realised within 10% of the nominal value for the lowest level and up to 1% variation at the highest. The method developed therefore proved to be robust and enough sensitive to attain LOQ in accordance with environmental quantification. To the best of our knowledge, this is the first method to apply MRM^3 quantification of small molecules in a complex environmental solid matrix. MRM^3 would then be useful to quantify small molecules at low concentration in environmental matrices.

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Figure captions

Figure 1: Repartition of the second-generation fragments with the modification of the applied AF2 energy (25 ms frag, dynamic LIT time, no Q0)

Figure 2: CEF fragmentation pattern in MRM3 acquisition, structure proposals from Metfrag (https://msbi.ipb-halle.de/MetFrag)

Figure 3: Repartition between 1st generation and 2nd generation fragments in function of the excitation time (AF2=0.1 V, Dynamic LIT time)

Figure 4: Signal increase noted for the second-generation fragment with the accumulation time increase (AF2=0.1 V, 25 ms excitation time)

Figure 5: Signal enhancement percentage when enabling Q0 trapping

Figure 6: Comparison of MRM and MRM^3 calibration curves of ceftiofur in sludge extracts

Figure 7: Extracted chromatogram of D5-Ampicillin in sludge extract: a) MRM transition, b) MRM^3 transition

Figure 1

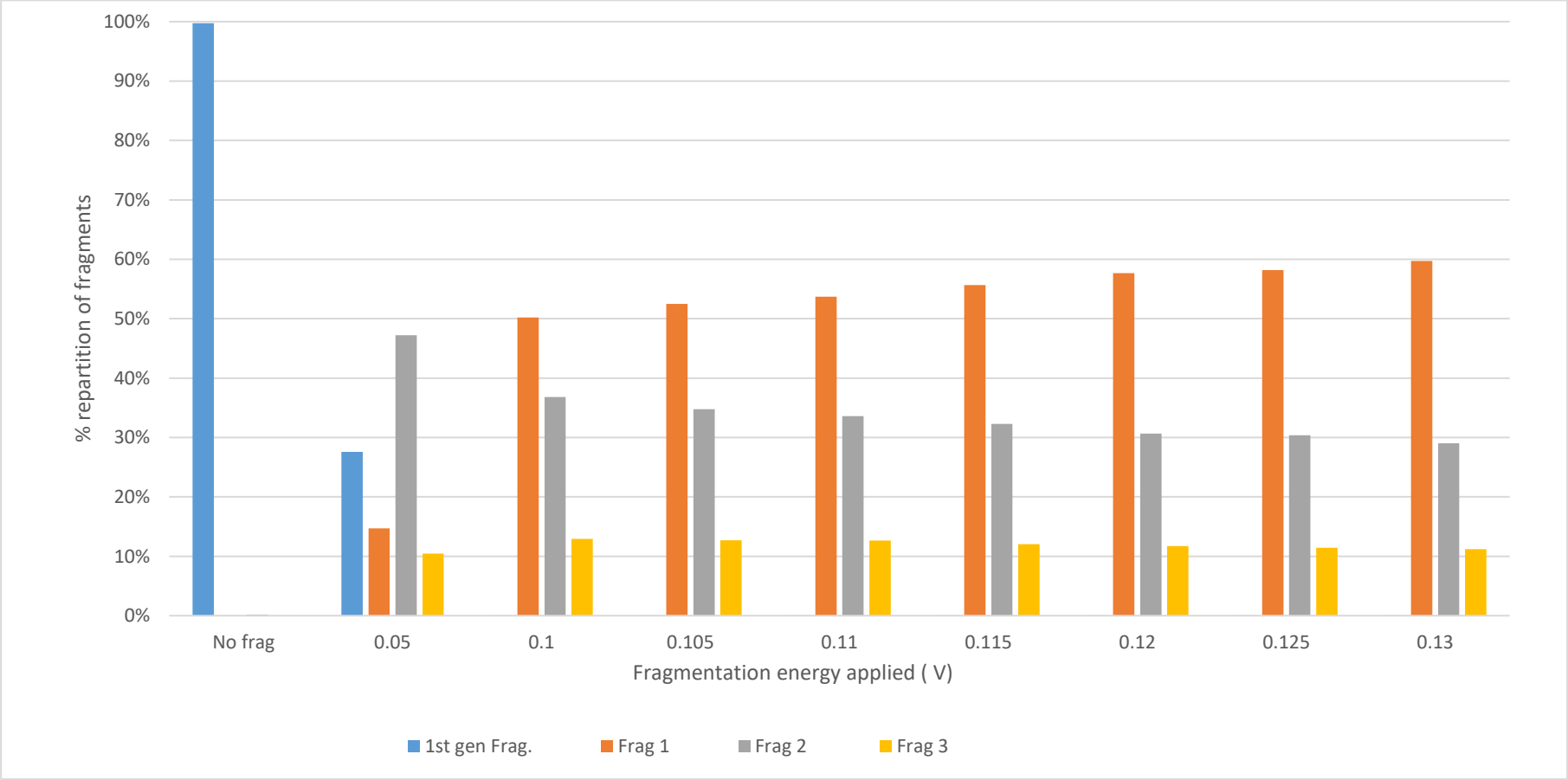
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
% repartition of fragments
No frag
0.05
0.1
0.105
0.11
0.115
0.12
0.125
0.13
Fragmentation energy applied (V)
1st gen Frag.
Frag 1
Frag 2
Frag 3

Figure 2

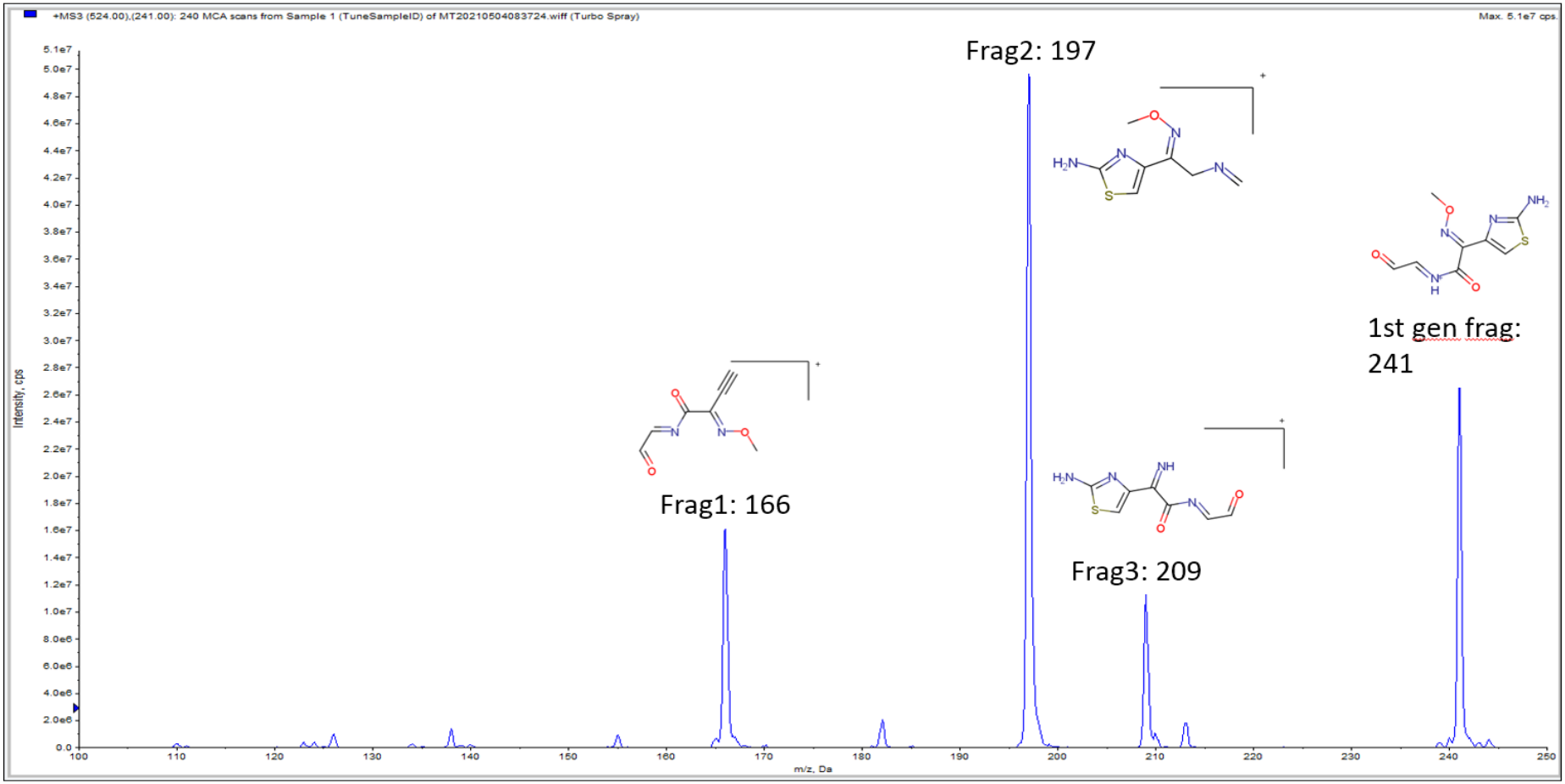
+MS3 (524.00),(241.00): 240 MCA scans from Sample 1 (TuneSampleID) of MT20210504083724.wiff (Turbo Spray)
Max. 5.1e7 cps.
Intensity, cps
m/z, Da
Frag2: 197
1st gen frag: 241
Frag1: 166
Frag3: 209

Figure 3

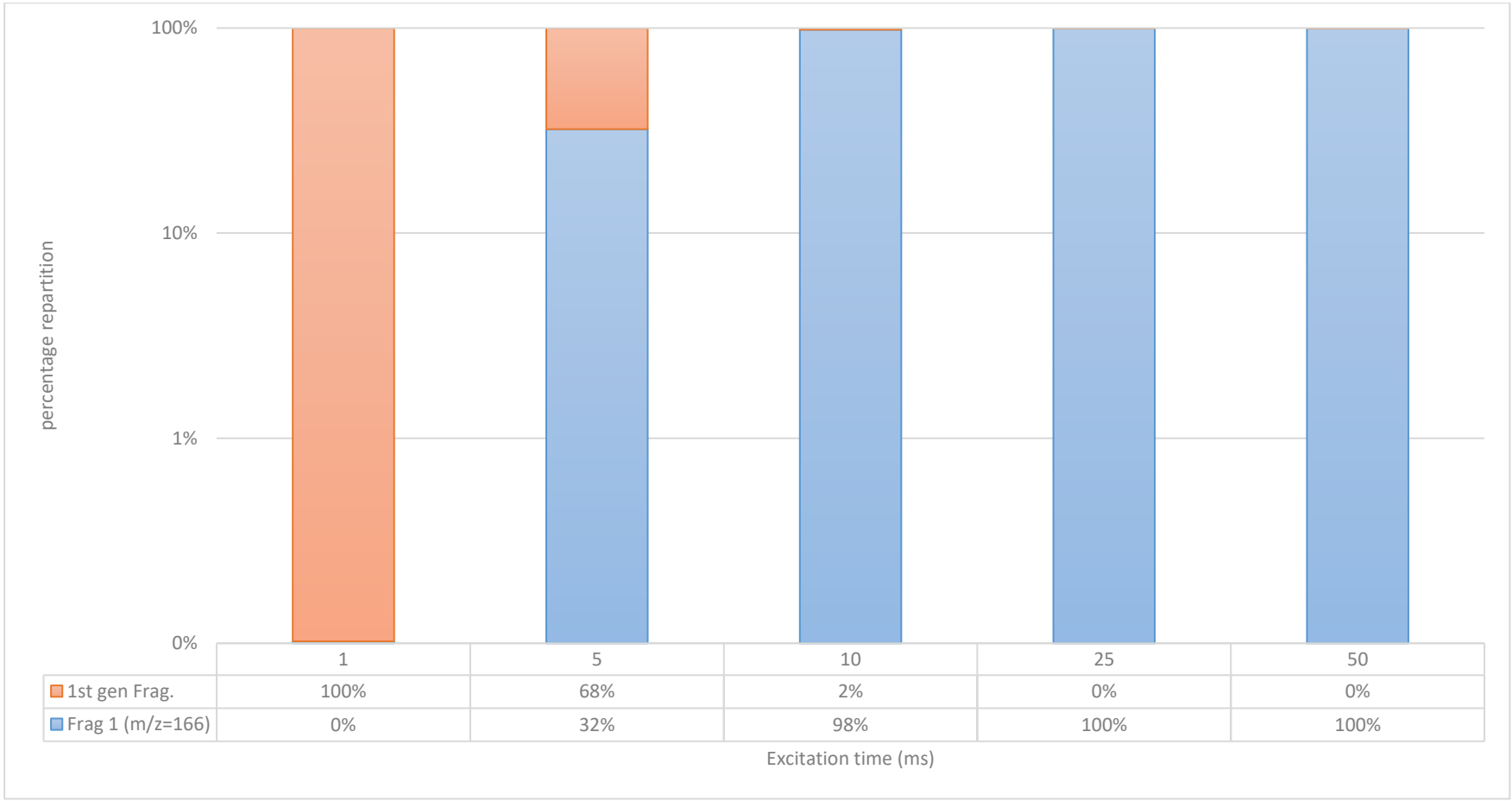
100%
10%
1%
0%
percentage repartition
1
5
10
25
50
1st gen Frag.
100%
68%
2%
0%
0%
Frag 1 (m/z=166)
0%
32%
98%
100%
100%
Excitation time (ms)

Figure 4

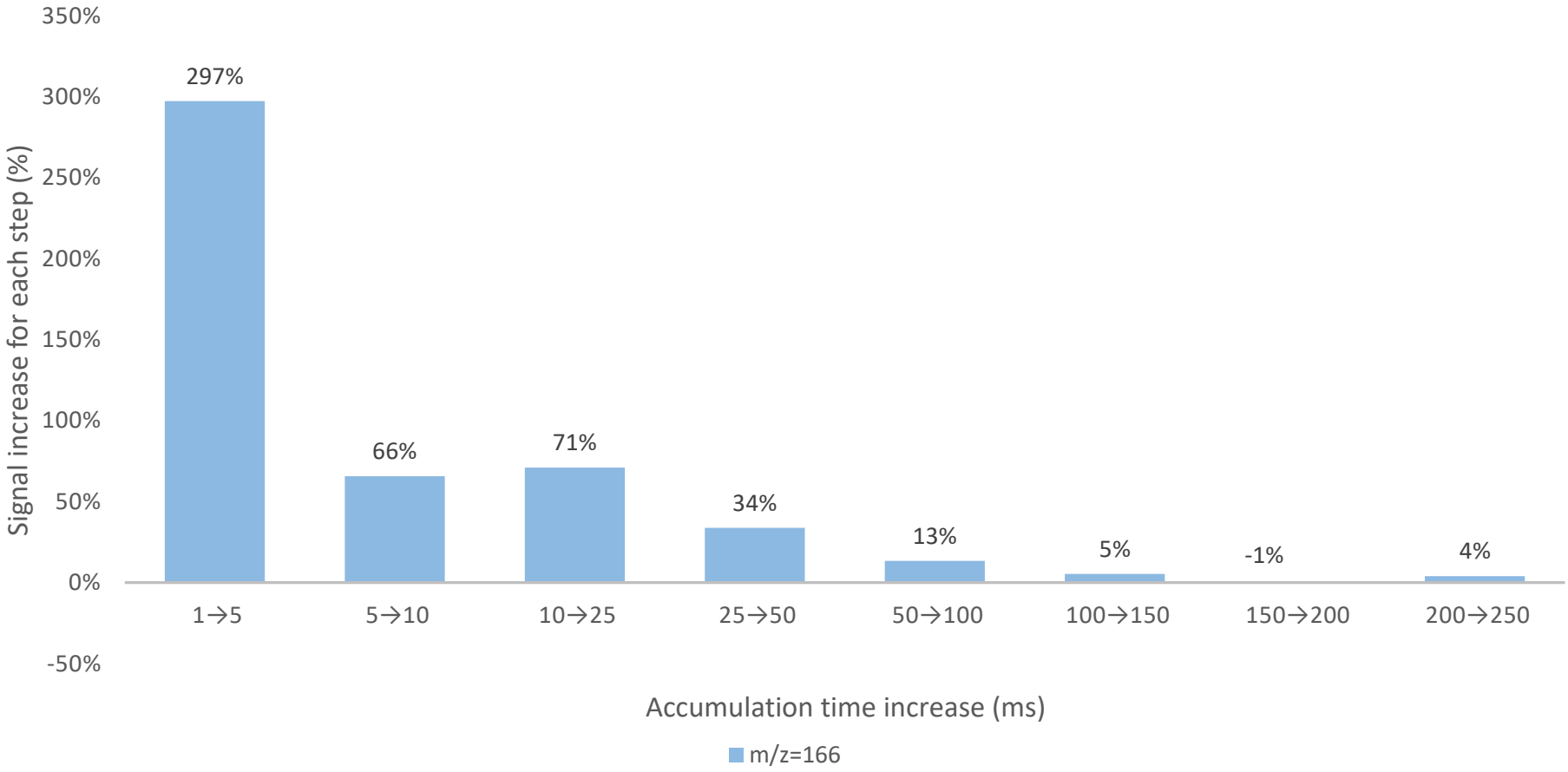
350%
300%
250%
200%
150%
100%
50%
0%
-50%
Signal increase for each step (%)
297%
66%
71%
34%
13%
5%
-1%
4%
1→5
5→10
10→25
25→50
50→100
100→150
150→200
200→250
Accumulation time increase (ms)
m/z=166

Figure 5

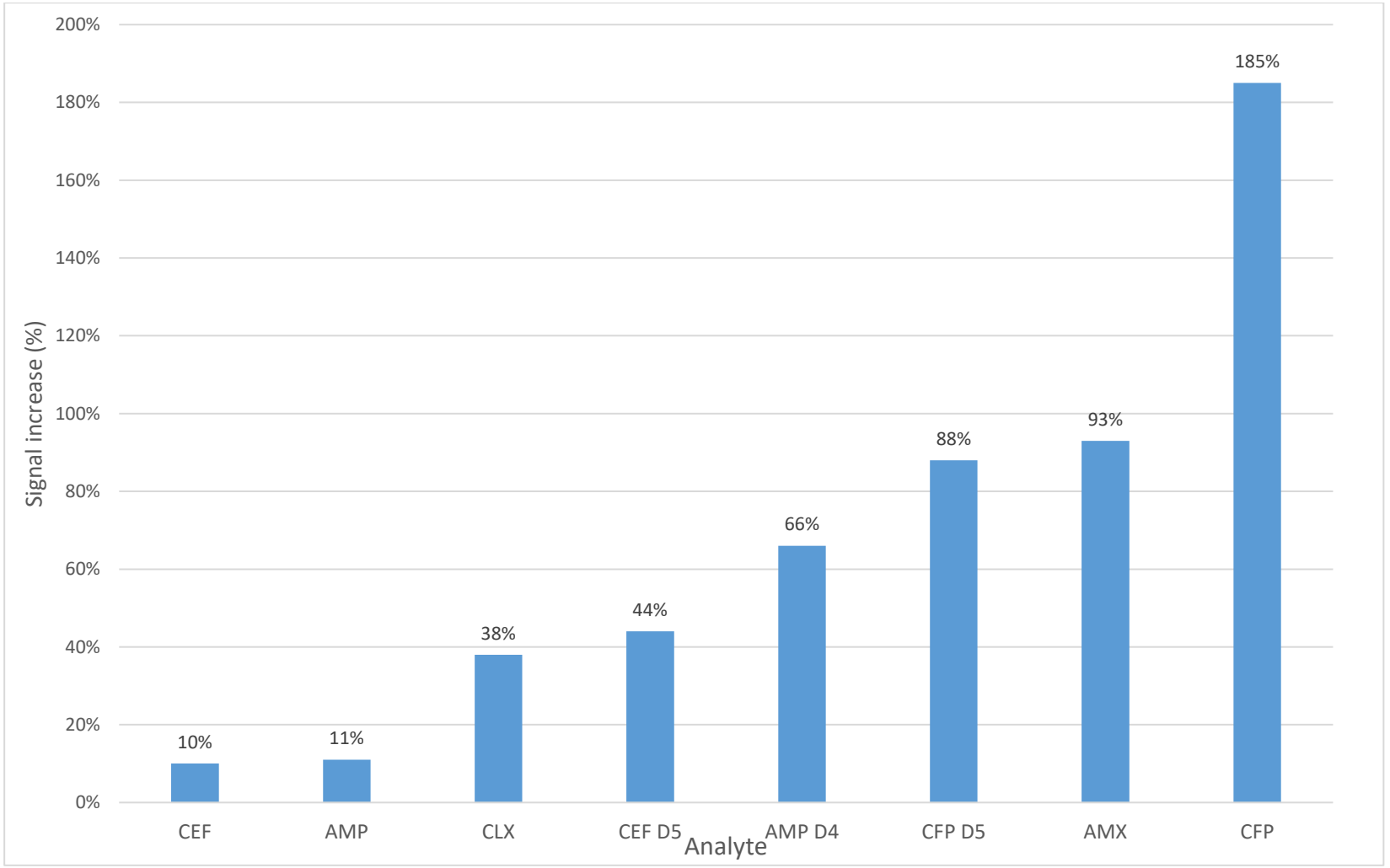
200%
180%
160%
140%
120%
100%
80%
60%
40%
20%
0%
Signal increase (%)
10%
11%
38%
44%
66%
88%
93%
185%
CEF
AMP
CLX
CEF D5
AMP D4
CFP D5
AMX
CFP
Analyte

Figure 6

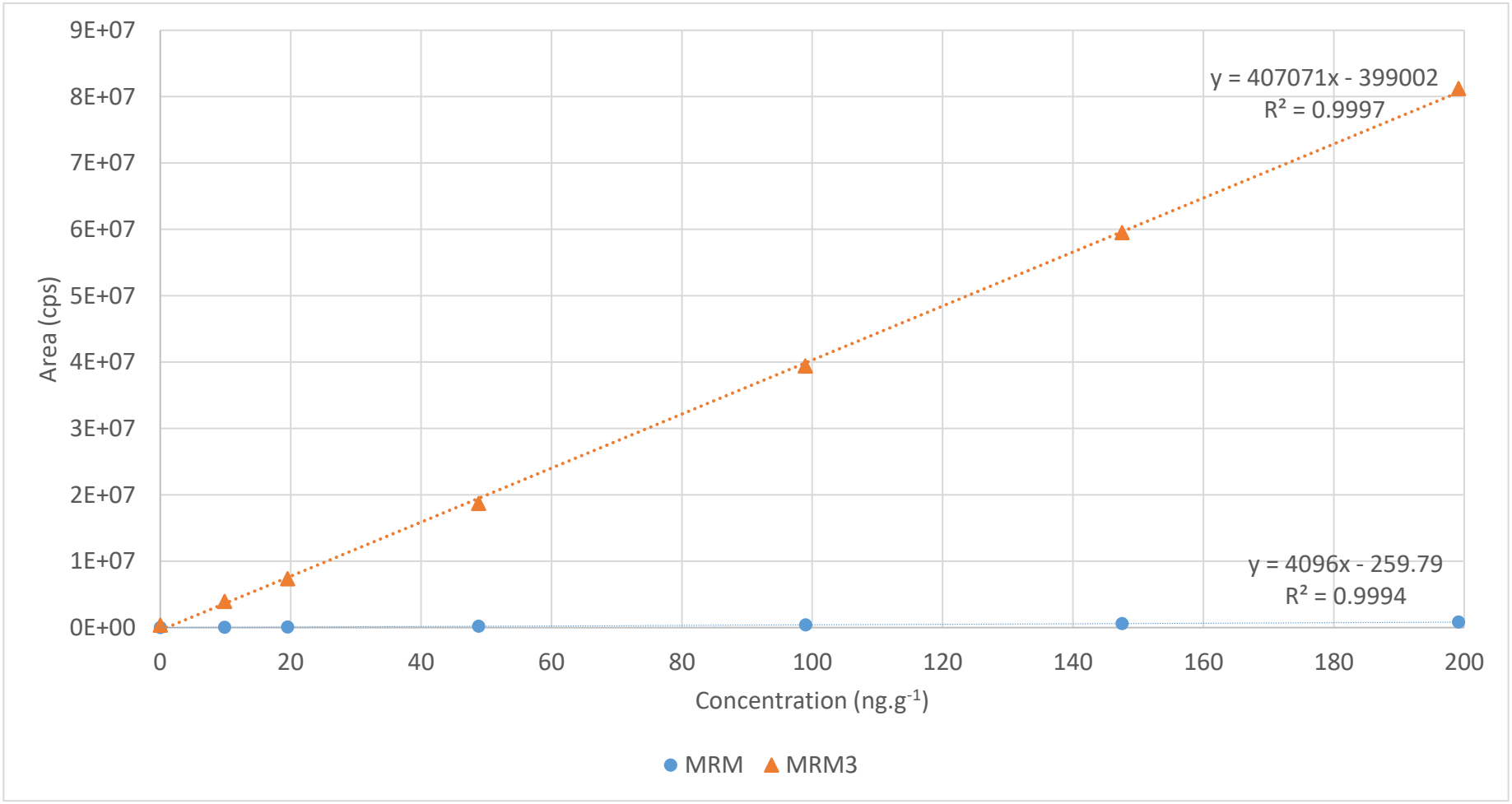
9E+07
8E+07
7E+07
6E+07
5E+07
4E+07
3E+07
2E+07
1E+07
0E+00
Area (cps)
0
20
40
60
80
100
120
140
160
180
200
Concentration (ng.g-1)
y = 407071x - 399002
R² = 0.9997
y = 4096x - 259.79
R² = 0.9994
MRM
MRM3

Figure 7

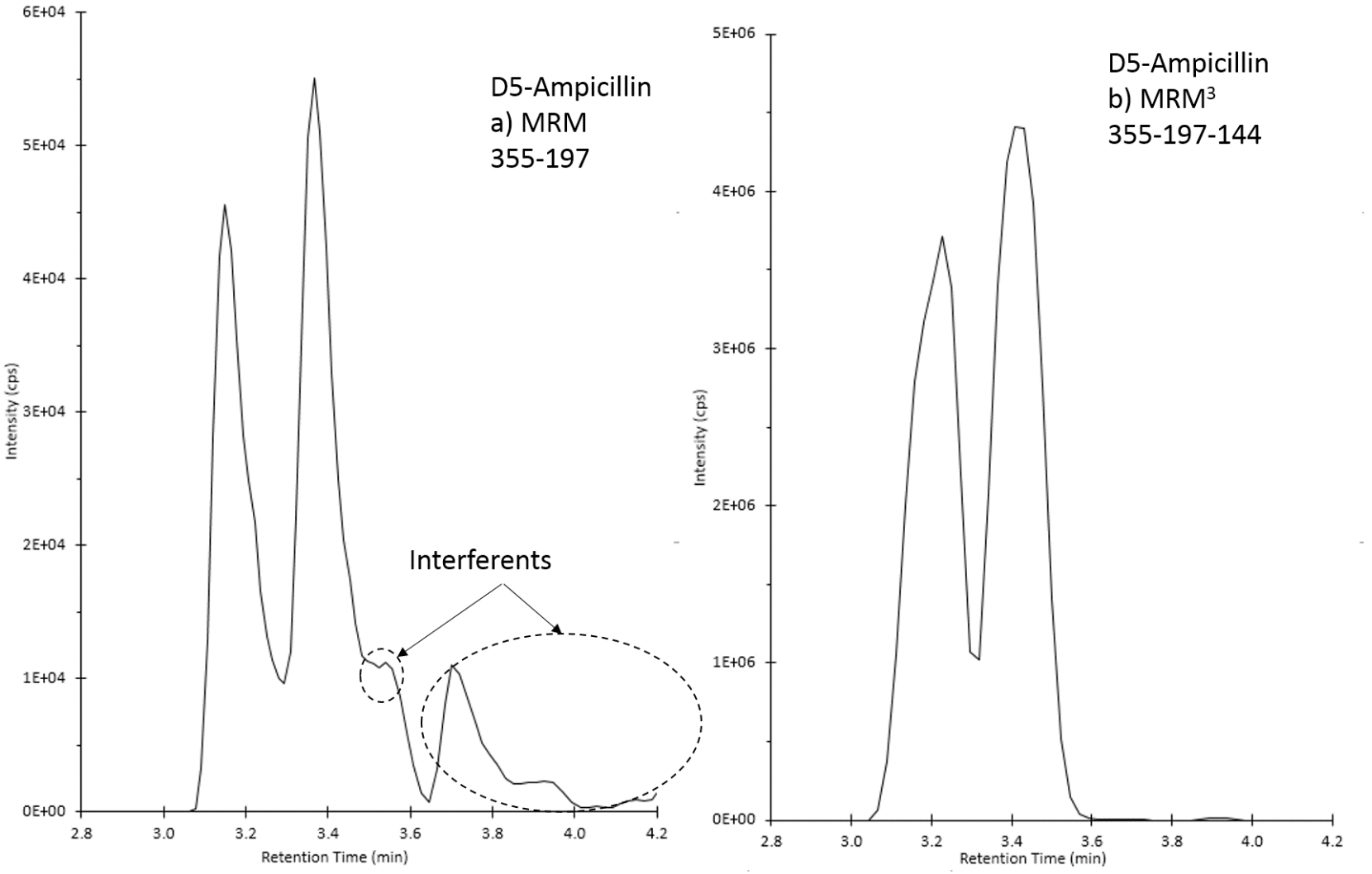
D5-Ampicillin
a) MRM
355-197
Interferents
Intensity (cps)
Retention Time (min)
6E+04
5E+04
4E+04
3E+04
2E+04
1E+04
0E+00
2.8
3.0
3.2
3.4
3.6
3.8
4.0
4.2
D5-Ampicillin
b) MRM3
355-197-144
5E+06
4E+06
3E+06
2E+06
1E+06
0E+00

Table 1: Overall optimisation gains in MRM^3 infusion acquisition

Analyte	**Non-optimised MRM^3 infusion intensity (cps)**	**Final intensity (cps) with optimised MRM3 parameters**	**Overall optimisation factor**
AMP	2.18E+08	1.00E+10	46
CEF	2.41E+08	8.73E+09	36
AMX	7.50E+08	2.90E+09	4
CFP	3.80E+06	9.70E+08	255
CLX	1.40E+07	1.80E+09	129

Table 2: Method validation results in LC-MRM^3 mode: linearity, LOQs, and precision at 3 different concentrations (a: intraday n=4; b: interday n=5 days)

	Retention time (min) (%RSD[b])	**LOQ (ng.g^{-1})**		**Linearity**		**20 ng.g^{-1}**		**100 ng.g^{-1}**		**200 ng.g^{-1}**	
		MRM^3	MRM	**Range (ng.g^{-1})**	**R²**	**Mean (%RSD)[a]**	**Accuracy (%RSD)[b]**	**Mean (%RSD)[a]**	**Accuracy (%RSD)[b]**	**Mean (%RSD)[a]**	**Accuracy (%RSD)[b]**
AMP	3.4 (1.1)	5.9	2.4	10-200	0.994	19.1 (15)	98 (15)	90.1 (17)	108 (12)	190.3 (6)	101 (6)
CEF	5.0 (1.0)	0.8	1.9	10-200	0.998	21.4 (11)	104 (12)	93.6 (3)	9+9 (8)	194.9 (1)	100 (1)
AMX	2.0 (3.1)	11.9	17.4	10-200	0.990	20.6 (17)	113 (13)	94.5 (10)	104 (12)	199.3 (11)	99 (10)
CFP	3.0 (1.3)	3.8	8.7	10-200	0.992	24.5 (17)	103 (10)	93.6 (4)	97 (9)	195.6 (10)	102 (4)
CLX	6.5 (0.8)	14.8	7.7	10-200	0.985	18.2 (25)	104 (6)	88.7 (10)	102 (11)	181.5 (7)	99 (5)

Table 3 : Sensitivity gain (computed with slopes ratio between MRM^3 and MRM) in LC-MRM^3 mode for both solvent and sludge extract

Analyte	**Signal intensity gain in solvent**	**Signal intensity gain in sludge extract**
AMX	29	6
AMP	12	6
CEF	123	95
CFP	79	63
CLX	52	3