Matrix effect compensation in small molecule profiling for a LC-TOF platform using multi-component post-column infusion

Oskar González[†‡], Michael van Vliet[†], Carola W.N. Damen[†], Frans M van der Kloet[†], Rob J. Vreeken[†], Thomas Hankemeier[†]*

[†]Division of Analytical Biosciences, Leiden Academic Centre for Drug Research, Leiden University, Einsteinweg 55 2333CC, Leiden (The Netherlands)

[‡]Analytical Chemistry Department, Faculty of Science and Technology, University of the Basque Country (UPV/EHU), Sarriena s/n, 48940 Leioa (Spain)

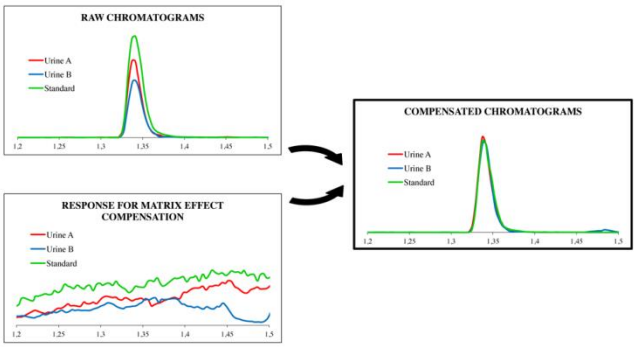
* oskar.gonzalezm@ehu.es / phone: +34946013366 / fax +34946013500

KEYWORDS: Matrix effect compensation, LC-MS, bioanalysis

ABSTRACT

The possible presence of matrix effect is one of the main concerns in LC-MS driven bioanalysis due to its impact on the reliability of the obtained quantitative results. Here we propose an approach to correct for the matrix effect in LC-MS with electrospray ionization using post-column infusion of eight internal standards (PCI-IS). We applied this approach to a generic

UHPLC-TOF platform developed for small molecule profiling with a main focus on drugs. Different urine samples were spiked with 19 drugs with different physicochemical properties and analyzed in order to study matrix effect (in absolute and relative terms). Furthermore, calibration curves for each analyte were constructed and Quality Control samples at different concentration levels were analyzed to check the applicability of this approach in quantitative analysis. The matrix effect profiles of the PCI-ISs were different: this confirms that the matrix effect is compound dependent, and therefore the most suitable PCI-IS has to be chosen for each analyte. Chromatograms were reconstructed using analyte and PCI-IS responses, which were used to develop an optimized method which compensates for variation in ionization efficiency. The approach presented here improved the results in terms of matrix effect dramatically. Furthermore, calibration curves of higher quality are obtained, dynamic range is enhanced and accuracy and precision of QC samples is increased. The use of PCI-ISs is a very promising step towards an analytical platform free of matrix effect, which can make LC-MS analysis even more successful, adding a higher reliability in quantification to its intrinsic high sensitivity and selectivity.



Introduction

Liquid chromatography-Mass Spectrometry (LC-MS) is currently the most widespread analytical technique in different fields such as environmental analysis, food analysis, bioanalysis or metabolomics[1-3]. The introduction of this technique resulted in a great advance in terms of sensitivity and selectivity, especially when tandem mass spectrometry (MS/MS) was used. The superior selectivity afforded by mass spectrometry detectors allowed the development of analytical methods with minimal sample treatment and high-throughput analysis since complete LC separation was not the only discriminating factor. Nevertheless, the analytical community realized that coeluting compounds could affect the ionization of the analyte, even if they were not identified as interferences affecting the selectivity of the method. The effect in the ionization, which can be either positive (ion enhancement) or negative (ion suppression) is known as matrix effect and is currently one of the main issues in LC-MS, especially from a quantitative point of view. Although many theories have been proposed to explain matrix effect[4-8], the exact mechanisms remain yet unexplained. It is well known that the ion suppression/enhancement process takes place in the ionization source and consequently both the type of ionization source employed and its design affect matrix effect[9,10]. In this sense, ElectroSpray Ionization (ESI) is prone to matrix effect due to its ionization mechanism[11-13]. It is also well known that the ionization efficiency of the analyte is highly dependent on its physicochemical properties: on the one hand molecules with high ionization potential are more easily ionized and on the other hand molecules with high surface affinity have more access to the excess of charge in the surface of the droplet. In the same way, coeluting compounds can affect the ionization process to a different extent either in the liquid phase by hampering droplet formation, impeding solvent desolvation or competing for the excess of charge or in the gas phase via proton exchange.

The occurrence of matrix effect in quantitative LC-MS analysis can severely affect the reliability of the results. For instance, if the signal of the analyte of interest is suppressed the slope of the calibration curve will be reduced and therefore the sensitivity of the method will be affected; which can result in analytes close to the lower limit of quantitation (LLOQ) not being detected. In any case, the main challenge associated with matrix effect is the sample to sample variability which can result in multiple problems: inaccurate quantitative results if the sample and the calibration standards have different matrix effect, false positives/negatives if the internal standard (IS) is more suppressed/enhanced in the sample, etc. Any of these cases leads to unreliable quantification and could be overlooked, therefore matrix effect has to be carefully studied. In this respect, two different concepts can be distinguished: absolute matrix effect and relative matrix effect. The difference in response between a matrix sample and a sample without coeluting compounds (solvent sample) is defined as absolute matrix effect and is not considered a critical parameter as long as it is consistent among different samples of the matrix of interest. The latter rarely happens with complex biological matrices where the amount and nature of the coeluting compounds change from one sample to another increasing the variability of the response. This variation, expressed as the Relative Standard Deviation (RSD) of the absolute matrix effect between samples of the same matrix is known as relative matrix effect. According to main bioanalytical validation guidelines this is a parameter to be studied during method validation and its limit is usually set at 15%[14-16].

Whenever the matrix effect can affect the results of the analysis, it must be properly eliminated or compensated. The ideal way to avoid matrix effect is the elimination of all the coeluting compounds that affect the signal of the analyte, which can be achieved by using a more exhaustive sample treatment/clean-up or by improving the chromatographic separation.

Nevertheless, this is only possible when the analytical method is focused on the analysis of a few analytes or analytes with very similar physicochemical properties; for multicomponent analysis it is hardly possible to develop a specific sample treatment method without a significant decrease of the recovery of some analytes and/or to separate all the coeluting compounds from the different analytes. When the coeluting compounds cannot be eliminated, there are several approaches to compensate or minimize the impact of the matrix effect such as standard addition calibration, sample dilution[17] or extrapolative dilution[18] and echo peak technique[19-22]. Probably the most widely accepted approach to combat the matrix effect is the use of analogue isotopically labelled internal standards (IL-IS). If an analysis is carried out using one IL-IS for several related compounds, as is often done, the internal standard correction will only work if the matrix effect is constant along the chromatographic run[23]. However, this is not often the case and consequently the matrix effect can be extremely different for the IL-IS and other analytes of interest due to the high retention time dependence of matrix effect. In order for this approach to work, an IL-IS should be used for each analyte (eluting at the same retention time) which is not always possible due to the cost and lack of availability of IL-IS for certain compounds. Furthermore, some authors described cases were the IL-IS was not able to correct for the matrix effect of the analyte due to slight changes in retention time or extraction recovery caused by deuterium isotope effect[24,25].

An innovative approach that can remove the retention time dependence is to infuse the IL-IS or a physicochemically related compound continuously after the chromatographic separation. Post-column infusion has been widely used as a qualitative assay of matrix effect since Bonfiglio[26] introduced it, but it has not been thoroughly applied to quantitative assays[27-30]. Stahnke *et al.*[31] studied the matrix effect profile of 129 pesticides in 20 different plant matrices using a triple

quadrupole (QQQ) instrument. They found a high dependence in the matrix effect profile on the plant matrix but very similar behavior of the different analytes within the same matrix, thus they used a single pesticide as post-column infusion internal standard (PCI-IS) and corrected the signal of each analyte using the average of the matrix effect of the IS around the retention time of the analyte. A significant improvement in terms of absolute matrix effect was obtained for almost all the analytes in the different matrices, although the compensation was not completely effective due to the fact that a single substance could not correct the matrix effect of all the analytes to the same extent. Liao *et al.* successfully applied a post-column infusion compensation method with an improved data acquisition for the quantification of 6 benzodiazepines in urine using a single PCI-IS chosen among 4 different candidates[32]. More recently the same group developed an analysis method for etoposide and etoposide catechol in two different types of biological matrices. The PCI-IS alone did not compensate in the same way for different matrices, therefore a second correction using a Matrix Normalization Factor (MNF) was introduced in order to normalize for the different absolute matrix effects in these two biofluids[33].

Here we propose a LC-MS method with a continuous post-column infusion approach using eight PCI-ISs simultaneously in order to develop a generic methodology for the compensation of the matrix effect in urine. Due to the high variability in composition this matrix is ideal to study the efficiency of the proposed compensation approach. Time Of Flight Mass Spectrometry (TOF) allows monitoring of as many m/z traces as necessary and the use of several PCI-ISs helps to correct for the absolute and relative matrix effect of analytes with very different physicochemical properties while offering valuable information about the matrix effect profiles. The data-processing involves reconstructed chromatograms for each analyte by doing a point by point correction using the most suitable PCI-IS. This approach significantly improves not only the

absolute matrix effect without further need of MNF but also the relative matrix effect, the dynamic range and the robustness of the method.

Experimental Section

Standards. Individual stock solutions of 1000 mg/L were prepared for each analyte in the most suitable solvent (water, methanol or acetonitrile) and properly diluted to 25 mg/L to make intermediate solutions. A multi-analyte working solution was prepared in methanol using the intermediate solutions to achieve a 1mg/L acetaminophen, candesartan cilexitil, clomipramine, diclofenac, digoxin, lacidipine, leucine enkephalin, nifedipine, telmisartan, simvastatin and 0.5 mg/L atenolol, betaxolol, bisoprolol, carteolol, labetalol, metoprolol and propanolol solution. An independent methanol solution at 0.5 mg/L levobunolol was also made. More information about Reagents and Chemicals is given in Supporting Information.

Study samples. In order to prepare the spiked urine samples (matrix samples) for matrix effect evaluation, 100 µL urine were transferred to an Eppendorf tube (Hamburg, Germany), 450 µL water and 250 µL methanol were added and the sample was spiked with 100 µL of multi-analyte working solution and 100 µL levobunolol solution. Samples were then centrifuged in an Eppendorf 5415 R centrifuge (5 min, 10000 g) and transferred to amber vial for LC-MS analysis. Solvent samples were prepared following the same procedure but replacing the urine by water. In this way all the samples had the same aqueous:methanol proportion (55:45) and a final concentration of 0.1 mg/L candesartan cilexitil, clomipramine, diclofenac, digoxin, lacidipine, leucine enkephalin, nifedipine telmisartan, acetaminophen, simvastatin and 0.05 mg/L atenolol, betaxolol, bisoprolol, carteolol, labetalol, levobunolol, metoprolol and propanolol solution.

For the study of the applicability of the post-column infusion approach to quantitative analysis, calibration standards containing all the previous analytes plus caffeine were prepared in a single urine source (aqueous phase:methanol (55:45)) at 3, 15, 30, 50, 100, 200, 350, 500, 750 and 1000 μg/L, except for levobunol, the concentration of which was kept at 50 μg/L to be studied as a IS. QC samples were prepared in urines from other three different sources at three different concentration levels (15 μg/L, 100 μg/L, 750 μg/L), in order to study accuracy and precision at least at two concentration levels.

LC-MS platform and post-column infusion set-up. Analyses were performed on a LC-MS platform intended for untargeted metabolomic profiling and quantitative and qualitative analysis of more than 80 drugs and drug metabolites. The platform consist of a Waters Ultra Performance Liquid Chromatograph (UPLC) coupled to a TOF instrument (Waters Synapt G2-S) with a scan range of 50-850 m/z. Chromatography was performed in gradient mode with a total run time of 5 minutes on a Waters Acquity UPLC HSS T3 column (1.8 μm, 2.1 x 50 mm) using as aqueous mobile phase a 0.01% HCOOH solution (A) and acetonitrile (B) as organic modifier. For the post-column infusion, the IntelliStart pumping system of the Synapt G2-S instrument was used. PCI-IS solution infusion (10 μL/min) was combined with the LC flow after chromatographic separation. Full details of the LC-MS platform are given in Supporting Information.

Data processing and evaluation of matrix effect. Masslynx 4.1 SCN916 (Waters, Wilmslow, UK) was used to acquire all the data which was stored as .raw folders. Using the *msaccess* tool included in ProteoWizard[34] open source software (v 3.0.6002), the m/z trace for each analyte and PCI-IS was extracted (tolerance window ±12.5 mDa). Reconstructed chromatograms were generated for each analyte by dividing the intensity at each time point by the intensity at the same time point for each of the PCI-IS m/z traces. Finally, the area under the chromatographic

peak was calculated for each of the analyte/PCI-IS chromatogram combinations. This approach corrects for the matrix effect at each specific time point of the chromatographic peak, which is significantly better than the approaches based on calculating the area of the analyte and correcting it using the average matrix effect of the PCI-IS around its retention time[31].

In order to evaluate the absolute matrix effect, the calculation defined by Matuszewski *et al.*[35] was used, where the matrix effect is expressed as the ratio (in percentage) of the response of the analyte in the matrix (matrix sample) over the response in a matrix free solution (solvent sample). In this way, values over 100% reflect ion enhancement whereas values below 100% reflect ion suppression. When the absolute matrix effect was calculated for different matrix sources, the average of all the values was used to reflect the average absolute matrix effect. Relative matrix effect, as the variation of matrix effect values of different sources of the same matrix, was calculated as the RSD of the absolute matrix effect of the different sources[14-16]. The comparison of these parameters for the original uncompensated data with the processed compensated data was used to evaluate the suitability of the proposed approach for matrix effect compensation.

Analytes of interest, PCI-ISs and their concentration for post-column infusion. In order to study the matrix effect compensation in the most extensive way, 19 compounds were chosen from the target list of a drug screening LC-MS platform. Eight of these compounds (atenolol, betaxolol, bisoprolol, carteolol, labetalol, levobunolol, metoprolol and propranolol) belong to the β-blocker family, thus they are suitable for studying the effectiveness of using only one PCI-IS for structurally related compounds. The other 11 analytes have very different physicochemical and cover a broad chromatographic range with acetaminophen eluting early in the chromatogram (1.38 min) and simvastatin at the very end of the gradient (3.19 min). Furthermore, they have

different MS ionization behaviour forming protonated molecular ions, Na and K adducts or in-source fragments. In order to find PCI-ISs that could be used to correct for the matrix effect of molecules with such a wide range of characteristics, 8 isotopically labelled analogues were chosen as PCI-ISs for the post-column infusion. In Table S-1 all the different analytes and PCI-ISs are depicted together with their molecular weight, chemical formula, retention time and the most intense ions that can be observed in the MS spectra. All the ions that are shown in this table were evaluated to find the best matrix effect compensation.

The concentration of the PCI-ISs in the post-column infusion solution is a crucial factor for proper matrix effect compensation: if the concentrations of the PCI-ISs are too high they will induce ion suppression and the sensitivity of the method will be affected. If the concentrations are too low the PCI-ISs will not reflect properly the matrix effect profile due to too much background noise. The concentrations chosen for the PCI-IS solution were 0.025 mg/L atenolol-d_7, 0.125 mg/L caffeine-d_3, 0.25 mg/L diclofenac-$^{13}C_6$, 0.030 mg/L lacidipine-$^{13}C_8$, 0.030 mg/L metformin-d_6, 0.125 mg/L nifedipine-d_6, 0.125 mg/L simvastatin-d_6 and 0.25 mg/L acetaminophen-d_4.

Matrix effect profiles and preliminary choice of PCI-IS. The term matrix effect profile was introduced by Stahnke *et al*[31]. and is used for the visual evaluation of the matrix effect of an analyte in a certain matrix over the whole chromatographic range. While the infusion profile shows the profile of the analyte signal over the chromatographic range, the matrix effect profile combines the infusion profiles of a matrix sample and a solvent sample (in the same way as for the matrix effect calculation) so ion suppression and enhancement areas can be easily identified.

In order to study the matrix effect profiles and choose the most suitable PCI-IS, solvent samples (n=3) and matrix samples (n=3) using urines from three different volunteers were used. Relative matrix effect and absolute matrix effect average were calculated for each analyte (considering all the different ions) using all the different PCI-ISs. Furthermore, in order to find the best PCI-IS for each target ion in a fast and intuitive way, the matrix effect for the different urines were plotted versus the PCI-IS used for the compensation.

Study of matrix effect compensation in urine. Once the potential PCI-IS candidates were chosen, a larger batch of urine samples was analyzed. A total of 15 spiked urines from different individual volunteers (different age, BMI, sex...) were analyzed in triplicate together with 9 different solvent samples. After LC-MS analysis and data preprocessing, the relative matrix effect and absolute matrix effect average were calculated for each analyte using the potential PCI-ISs.

Application of the post-column infusion approach to quantitative drug analysis. The main goal of a bioanalytical method is the reliable quantitation of drugs or metabolites in a biological matrix. As mentioned earlier, the high variability in kind and amount of endogenous compounds in matrices from different sources usually is accompanied with a high relative matrix effect which can lead to unreliable results. Urine is one of the most common biological matrices for drug screening and shows a high variability in matrix effect (the composition is less regulated than in plasma), making it a suitable matrix to study the proposed compensation approach. Additionally, the determination in urine of many of the compounds used for this study can present an analytical challenge (especially acetaminophen, caffeine and β-blockers), thus, the applicability of the post-column infusion approach to quantitative analysis of the analytes of interest was studied. For this aim, urine calibration standards ranging from 3 to 1000 µg/L and

the QC samples prepared in urines from other three different sources at 15 μg/L, 100 μg/L and 750 μg/L were analyzed.

Firstly, linearity range was defined for compensated and uncompensated calibration curves between the first calibration standard offering a S/N higher than 10 and the calibration standard where the curve loses the linearity. These results were also used to study the enhancement of the dynamic range when matrix effect compensation is applied.

In order to study precision and accuracy, RSD and Relative Error (RE) to the theoretical concentration value for the different QC samples at the different concentration levels were compared before and after applying matrix effect compensation (at least two QC samples fit into the calibration curve in order to measure these figures of merit at two different levels). Furthermore, these parameters were also calculated for all the compounds using using levobunolol as a traditional IS (by correcting analyte area with levobunolol area). Caffeine was also included in these experiments in order to study the applicability of standard addition calibration. Although the caffeine concentration of the samples is unknown, the addition of the same amount of caffeine to each sample would produce an equal increase in signal in the absence of matrix effect. This means that the slope of calibration curves built by standard addition in different urine samples would remain constant and therefore this parameter can be used to study the effectiveness of matrix effect compensation.

Results and Discussion

Matrix effect profiles and preliminary choice of PCI-IS. Matrix effect profiles for the different PCI-ISs were studied and shown to be very different for the same sample and LC-MS run. Atenolol-d_7, caffeine-d_3 and acetaminophen-d_4 on the one hand (moderately polar

compounds), and lacidipine-$^{13}C_8$ and simvastatin-d_6 on the other hand (most nonpolar compounds), were the only PCI-ISs to show similar matrix effect profiles. There were not only significant differences among the profiles of the different PCI-ISs, but also between the Na and K adducts of the same analyte (lacidipine-$^{13}C_8$, nifedipine-d_6 and simvastatin-d_6). Therefore, different PCI-ISs and different m/z ratios to compensate for the matrix effect are obviously necessary. In Figure 1, the difference between metformin-d_6 and caffeine-d_3 ($[M+H]^+$ ions) matrix effect profiles can be clearly observed suggesting different ionization behavior. Although both molecules suffer from high ion suppression in the first two minutes of the chromatogram, this effect is more pronounced for metformin-d_6. From that point onwards the suppression decreases, which can be explained by the high amount of salts and other polar endogenous compounds in urine eluting early in the chromatogram.

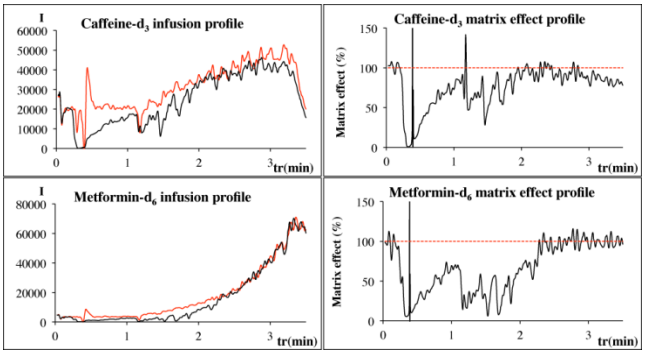


Figure 1. Infusion and matrix effect profiles for caffeine-d_3 $[M+H]^+$ (above) and metformin-d_6 $[M+H]^+$ (below) in a blank solvent solution (red) and a blank urine sample (black). The red line in the matrix effect profile chart shows the 100% matrix effect line.

In order to choose the best PCI-IS in a fast and reliable way, the absolute matrix effect calculated for each analyte in the 3 urines was plotted against the different PCI-ISs (including the results obtained without compensation) with the error bands showing the standard deviation calculated

from the replicates of the matrix and the solvent samples. A suitable PCI-IS is the one that results in an absolute matrix effect of 100% (±15%) and a relative matrix effect smaller than 15%. Figure 2 shows an example of this chart for carteolol. It can be immediately observed that atenolol-d_7, diclofenac$^{13}C_6$, nifedipine-d_6 $[M+Na]^+$ and acetaminophen-d_4 compensate for the absolute matrix effect (i.e. absolute matrix effect values of the urines are close to the ideal value of 100%) while atenolol-d_7 and acetaminophen-d_4 reduce also the relative matrix effect (i.e. absolute matrix effect values for the different urines are closer to each other). Taking these results into account, atenolol-d_7 and acetaminophen-d_4 are the first choice PCI-ISs to be used for carteolol compensation. For those PCI-ISs which do not offer a proper absolute matrix effect compensation, Liao *et al.*[33] proposed a normalization using MNF (a factor to be calculated and applied for each different matrix in order to normalize the matrix values to the standard values). In our experience, it appears not to be necessary if a multi-component PCI-IS solution is used where a PCI-IS can be found for each analyte that corrects for absolute matrix effect (i.e. MNF is 1). In this way, an additional correction of the already corrected data is avoided. Furthermore, a PCI-IS which compensates for the absolute matrix effect is more reliable since it indicates more similar ionization/desolvation behavior which also means that the relative matrix effect will be better compensated. The fact that a MNF is not necessary suggests that the method could be applied to different matrices to correct absolute matrix effect without additional calculations. In order to evaluate this, the proposed method was applied to plasma samples spiked with carteolol after a simple protein precipitation procedure with methanol (1:3 plasma:methanol). Ion suppression was also observed for carteolol in plasma, but it was less severe (~80%) than in urine samples (~60%); like for urine, atenolol-d_7 (and acetaminophen-d_4) offered excellent compensation for plasma as can be observed in Figure 3.

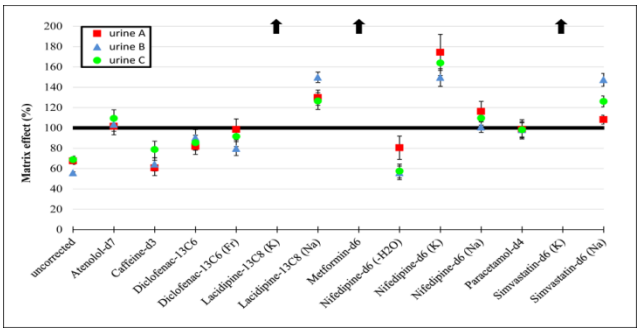


Figure 2. Chart for the selection of the most suitable PCI-IS for carteolol showing the absolute matrix effect for three different urines (urine A: squares, urine B: triangles, urine C: circles) for each PCI-IS together with their standard deviations (vertical error bands). Black arrows indicate absolute matrix effect values over 200%.

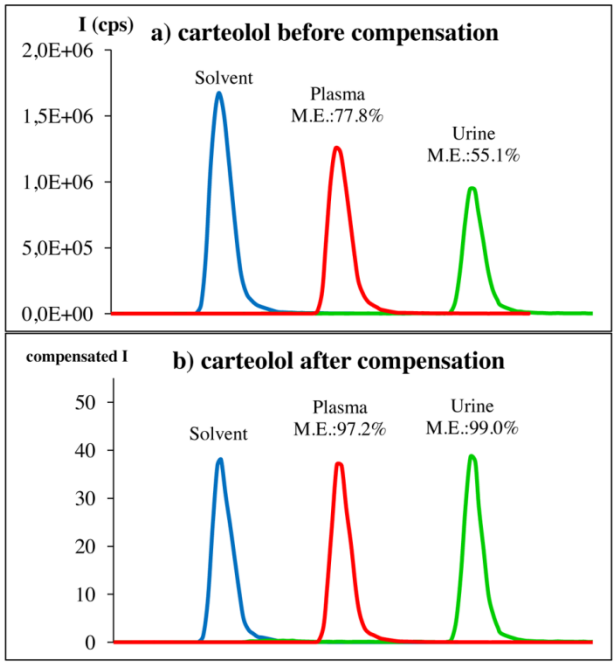


Figure 3. Chromatographic signals for carteolol in solvent (blue), plasma (red) and urine (green) before (a) and after compensation with atenolol-d_7 (b). Matrix effect (M.E) is successfully compensated for both matrices

Table 1 shows the ion used for the analysis of each analyte and its most suitable PCI-IS obtained following the proposed procedure. Other PCI-ISs that improve the results to a lesser extent are also indicated. When adducts were the most suitable ions for the analysis (lacidipine, nifedipine and simvastatin), only a single adduct was used. As expected the best compensation was obtained using PCI-IS that forms adduct with the same alkali metal. It is remarkable that the best PCI-IS for the β-blockers is not always the labelled β-blocker analogue (atenolol-d_7) but caffeine-d_3 or acetaminophen-d_4 (which show similar matrix effect profiles) These results are consistent with Sthanke *et al*. and Liao *et al.* observations[31-32]. The latter surprisingly found out that the most effective PCI-IS for the analysis of 6 benzodiazepines was not the structural analogue nordiazepam but a chemically unrelated fluorinated posphazene.

Table 1. Most suitable PCI-ISs and other potential PCI-ISs per analyte.

Analyte	**Best PCI-IS**	**Other suitable PCI-ISs**
Acetaminophen $[M+H]^+$	Acetaminophen-d_4 $[M+H]^+$	Atenolol-d_7 $[M+H]^+$, Diclofenac-$^{13}C_6$ $[M+H]^+$
Atenolol $[M+H]^+$	Atenolol-d_7 $[M+H]^+$	Nifedipine-d_6 $[M+Na]^+$,
Betaxolol $[M+H]^+$	Caffeine-d_3 $[M+H]^+$	Acetaminophen-d_4 $[M+H]^+$
Bisoprolol $[M+H]^+$	Caffeine-d_3 $[M+H]^+$	Acetaminophen-d_4 $[M+H]^+$
Candesartan cilexetil $[Fr+H]^+$	Simvastatin-d_6 $[M+Na]^+$	Diclofenac-$^{13}C_6$ $[Fr+H]^+$
Carteolol $[M+H]^+$	Atenolol-d_7 $[M+H]^+$	Acetaminophen-d_4 $[M+H]^+$, Nifedipine-d_6 $[M+Na]^+$
Clomipramine $[M+H]^+$	Atenolol-d_7 $[M+H]^+$	Acetaminophen-d_4 $[M+H]^+$, Nifedipine-d_6 $[M+Na]^+$
Diclofenac $[Fr+H]^+$	Diclofenac-$^{13}C_6$ $[Fr+H]^+$	Diclofenac-$^{13}C_6$ $[M+H]^+$, Metformin-d_6 $[M+H]^+$
Digoxin $[Fr+H]^+$	Diclofenac-$^{13}C_6$ $[Fr+H]^+$	Diclofenac-$^{13}C_6$ $[M+H]^+$, Caffeine-d_3 $[M+H]^+$
Labetalol $[M+H]^+$	Metformin-d_6 $[M+H]^+$	Nifedipine-d6 $[M+Na]^+$
Lacidipine $[M+Na]^+$	Lacidipine-$^{13}C_8$ $[M+Na]^+$	Nifedipine-d_6 $[M+Na]^+$, Simvastatin-d_6 $[M+Na]^+$
Leucine enkephalin $[M+H]^+$	Diclofenac-$^{13}C_6$ $[M+H]^+$	Caffeine-d_3 $[M+H]^+$
Levobunolol $[M+H]^+$	Nifedipine-d_6 $[M+Na]^+$	Atenolol-d_7 $[M+H]^+$, Caffeine-d_3 $[M+H]^+$
Metoprolol [M+H]+	Caffeine-d_3 $[M+H]^+$	Diclofenac-$^{13}C_6$ $[Fr+H]^+$
Nifedipine $[M+Na]^+$	Nifedipine-d_6 $[M+Na]^+$	Lacidipine-$^{13}C_8$ $[M+Na]^+$, Simvastatin-d_6 $[M+Na]^+$
Propanolol $[M+H]^+$	Acetaminophen-d_4 $[M+H]^+$	Caffeine-d_3 $[M+H]^+$, Atenolol-d_7 $[M+H]^+$
Simvastatin $[M+Na]^+$	Simvastatin-d_6 $[M+Na]^+$	Nifedipine-d_6 $[M+Na]^+$, Lacidipine-$^{13}C_8$ $[M+Na]^+$
Telmisartan $[Fr+H]^+$	Diclofenac-$^{13}C_6$ $[M+H]^+$	Diclofenac-$^{13}C_6$ $[Fr+H]^+$

*Fr: In-source fragment

Study of matrix effect compensation in urine. After the application of the compensation, the absolute matrix effect values for the 15 different urine samples are close to 100% for most of the

analytes, which indicates the absence of absolute matrix effect. The correction effect is especially significant for analytes suffering from high ion suppression such as atenolol and carteolol. The relative matrix effect also improves when applying the compensation, which means that the inter-sample variability is reduced and therefore the analysis reliability is increased. Several analytes which showed a relative matrix effect above 15% (atenolol, bisoprolol, carteolol, labetalol and acetaminophen) before the compensation had improved performance characteristics after correction that allowed them to meet the criteria established for bioanalytical validation. The results obtained for absolute matrix effect (average of all the absolute matrix effect values) and relative matrix effect for the 15 urine samples before and after applying the matrix effect compensation are given in Table S-2. It is remarkable that the relative and absolute matrix effect for labetalol could not be compensated simultaneously by any of the PCI-IS, which means that no correct PCI-IS was included in the mixture. Since the relative matrix effect is a more critical parameter in quantitative analysis (as long as matrix-matched calibration curves are used) absolute matrix effect was sacrificed for a better sample to sample variability. This issue could be fixed by using a combined compensation with two or more PCI-IS.

In order to show the results obtained after matrix effect compensation for the 15 different urine samples in more detail, Figure 4 shows the peak area for carteolol after and before applying the ME compensation.

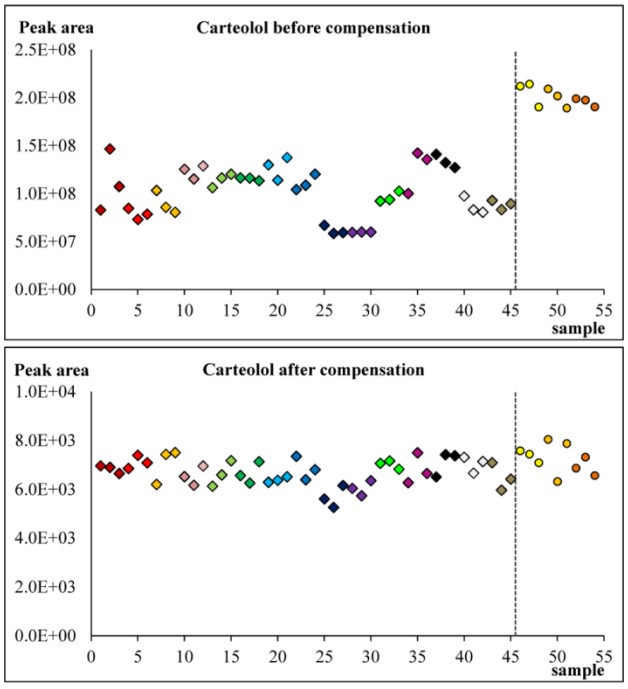


Figure 4. Carteolol area of for all the replicates of the 15 urine samples (1-45) and the solvent samples spiked at 50 µg/L (46-55). The upper chart shows data before applying the compensation and lower chart after correcting the signal using atenolol-d_7.

In the uncompensated data, the signal for all the matrix samples is lower than the signal of the solvent samples due to the ion suppression caused by the coeluting compounds. After applying the compensation, the difference in signal of the different urine samples becomes lower (improvement in relative matrix effect) and their signal is comparable to the solvent samples signal (improvement in absolute matrix effect). These results are also shown in terms of matrix effect in Figure S-1.

Application of the post-column infusion approach to quantitative drug analysis. The application of the post-column infusion compensation resulted in a significant improvement in terms of linearity and accuracy and precision of the QC samples. For the very polar compounds

atenolol and carteolol, which showed a high relative matrix effect in the previous experiments, this improvement is especially significant. For instance, before applying any correction, the RE for atenolol is as high as 73.7% while after applying post-column compensation this value is lower than 20% in all the cases. It was observed that the use of traditional IS correction improves the results to a lesser extent (maximum RE of 33.9%), which can be explained by the fact that levobunolol does not coelute with these compounds and therefore the matrix effect is different. Once again, the strong dependence of the matrix effect with the retention time is clear and it is proven that even if atenolol, carteolol and levobunolol are chemically related compounds, a single IS cannot be used for their reliable quantitation. On the other hand, metoprolol elutes close to levobunolol (1.55 vs 1.57 min) and therefore a good correction is obtained using only IS correction (highest RE decreases from 25.2% to 6.3% and RSD is reduced from 11.4% to 5.9% for 15 μg/L QC and from 7.5% to 3.7% for 100 μg/L QC). Nevertheless, in this case, the results obtained using post-column compensation also show a significant improvement compared to the uncompensated ones, which proves that PCI-IS can be an alternative approach to avoid the use of one IS per analyte in quantitative analysis. In Table S-3 the RE and RSD for the calibration standards and QC samples prepared in three different urine matrices at 2 concentration levels before applying any correction, after compensating for matrix effect and after correcting with levobunolol (IS) can be observed.

The aim of this study was to show the improvement in the analytical performance after correcting for the variability exclusively due to analyte ionization; therefore the sample preparation was kept as simple as possible. Obviously this is not the case for most analytical methods where a more complicated sample treatment is involved. In those cases, post-column compensation is to be combined with IS correction and obviously not only the analyte but also

the IS response must be compensated using the PCI-IS. This combined approach was also studied here, but since it did not affect the outcome, the results are not shown. Nevertheless, the fact that the IS concentration is constant in all the samples makes monitoring the IS response useful to control the performance of the compensation approach. In this case, the peak area of the IS decreases with increasing concentration of the analytes (Figure S-2), probably because co-eluting metoprolol and leucine encephalin cause more ion suppression. Compensation using atenolol-d_7 eliminated this trend, minimized the RSD (from 18.8 to 9.4%) and reduced the relative matrix effect (from 9.9 to 5.6%).

Finally, the application of the post-column infusion approach to standard addition calibration was studied using caffeine. An 11-point calibration curve was prepared for the urine used for calibration purposes and a point calibration for the QC samples. After matrix effect compensation, the RSD of the four slopes was reduced from 23.6 to 14.6%. Although the calibration points used were not homogeneously distributed, it is demonstrated that this approach can compensate for the matrix effect. If the slope of a calibration line is equal in all samples, standard addition calibration would not be longer necessary since one calibration curve could be used for the determination of the concentrations of all the samples, in other words, using the PCI-IS approach we do not need any more a time-consuming standard addition calibration.

Enhancement of dynamic range in LC-MS analysis. From the calibration curves built in order to study the applicability of the method to quantitative analysis we observed how the dynamic range for many analytes was increased, especially for lacidipine and simvastatin (Table S-3). These two compounds form very intense sodium adducts and elute at the end of the chromatogram where almost no ion-suppression is observed due to the lack of nonpolar coeluting compounds in urine (see Table S-2). In absence of matrix effect, a high accuracy and

precision was expected, but a calibration curve with an extremly short dynamic range was observed instead, as can be seen in Figure 5 for lacidipine. This recurrent problem can be due to the saturation of the detector (some TOF detectors have a limited dynamic range) or to an excess of ions on the droplet surface, so that not all the analyte molecules can be ionized and evaporated. The former can be easily identified by studying the isotopic pattern of the analyte; when the detector is saturated for a certain ion, the relative abundance of the M+1 and M+2 isotopic ions will increase. In this particular case, the ratio of the isotopic ions only showed a slight increase and the calibration curves built using the isotopic ions showed the same loss of linearity, so this problem could be probably explained by an excess of ions of the analyte of interest (or other coeluting analyte) in the droplet surface. The evaluation of the matrix effect profile is also useful to check whether the saturation is due to detection or matrix effect issues. By studying lacidipine-$^{13}C_8$ profiles of the different calibration standards, it was possible to observe how a suppression area around the analyte retention time appeared and becomes bigger as the concentration of the standards increased (Figure S-3), indicating analyte induced suppression and not MS detector saturation.

This phenomenon can be considered a self-induced matrix effect and therefore could be compensated by using the post-column infusion approach as can be observed in Figure 5. Due to the "self-induced ion suppression" the uncompensated calibration curve is not linear already after 30 µg/L (which makes impossible to build a calibration curve including more than three calibration standards) while the compensated caibration curve proved to be linear in the whole calibration range: acceptable RE for each calibration standard, random residual distribution and high coefficient of determination.

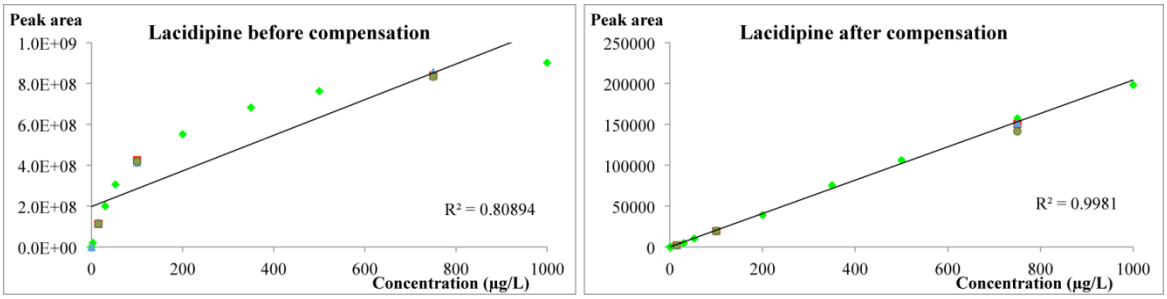


Figure 5. Calibration curve for lacidipine in urine before and after applying post-column compensation. Calibration standards (diamonds) and QC samples (squares, triangles and circles).

Some of the other analytes of interest show an improvement in the dynamic range when applying the compensation but the calibration curve is not linear for the whole calibration range. In these cases there is a combination of detector and ionization saturation as can be observed by building the calibration curve using the M+1 isotope of the analyte (Figure S-4). The dynamic range for the M+1 isotopic ion is broader (showing detector saturation) but still flattens at the highest points of the calibration due to ionization saturation. As in the previous cases, the latter effect can be compensated using the post-column infusion approach.

Conclusions

The multi-component post-column infusion approach we propose can successfully compensate for the matrix effect of a wide range of compounds in urine samples. We have shown that this approach can correct for relative matrix effect in different urine samples and thereby increase method precision and accuracy. We have also shown that the use of multiple PCI-IS allows us to correct for absolute matrix effect. This way, a second correction in the form of MNF is not necessary and suggests that a suitable PCI-IS for an analyte will likely be suitable for different matrices as was observed for plasma samples. Furthermore, the approach also allows correcting

for the ionization saturation caused by the excess of analytes in the samples, enhancing the dynamic range of the calibration curve.

The fact that this method corrects not only the relative but also the absolute matrix effect opens up new perspectives for LC-MS calibration for which matrix calibration is almost mandatory. If the correction for absolute matrix effect is properly validated, calibration curves can be built in solvent, saving time and making the analysis method more straightforward, especially in the case of rare or highly variable matrices. Post-column infusion could also mean a step forward in the analysis of endogenous compounds present in complex matrices. If the relative matrix effect is effectively compensated, the slope of all the calibration curves built using the different samples will be comparable and therefore there will be no need of building a calibration curve per sample. Furthermore, in absence of absolute matrix effect external calibration using solvent calibrators could be also feasible.

This is one of the few works where post-column infusion compensation has been applied to quantitative analysis using LC-MS and to our knowledge the first one using TOF detector. Although sensitivity and dynamic range of this detector is usually lower compared to QQQ, the sensitivity and dynamic range is good enough to realize PCI-IS correction as described, and it allows monitoring a whole m/z range which means an improvement compared to instruments working in multiple reaction monitoring mode, where the acquisition of the m/z traces of the PCI-ISs is limited by the dwell time and the number of analytes to be measured. Furthermore it opens a door to untargeted analysis (such as in metabolomics) since retrospective data processing is possible if the suitable PCI-IS is found in subsequent experiments The point by point correction and the generation of a reconstructed chromatogram using the most suitable PCI-IS for each analyte proved to provide a reliable correction. Although in the work we present here

the correction is performed using a single PCI-IS per analyte it would be possible to apply a multivariate correction method in order to represent the ionization behavior of the analytes in a more accurate way and to develop even a more generic method.

In contrast to results previously reported[31], we conclude that the use of single PCI-IS is not sufficient for matrix effect compensation of analytes with a wider range of physicochemical properties probably due to the fact that our analytes of interest belong to different compound classes. This means that there is also an analyte dependent matrix effect to be considered when choosing the proper PCI-IS and consequently the use of different PCI-ISs is necessary. We found that the choice of the PCI-IS based merely on (limited) a priori knowledge of the chemical structure is unsuccessful as was observed for the choice of the PCI-IS for β-blockers. In order to make an easy and fast empirical choice of the best PCI-IS per analyte, we proposed for the first time an intuitive method based on a chart plotting matrix effect versus the different PCI-IS.

In conclusion, the work presented here is a major step towards a matrix effect free LC-MS generic platform which adds a higher reliability and dynamic range to the intrinsic high sensitivity and selectivity of this analytical technique.

SUPPORTING INFORMATION

Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding author. Email: oskar.gonzalezm@ehu.es

Notes. The authors declare no competing financial interest.

ACKNOWLEDGMENTS

Oskar González thanks the Basque Government for his post-doctoral grant and Mª Encarnación Blanco for her help with the artwork. The project was also supported by the EU FP7 project STATEGRA (grant agreement nº 306000).

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