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Analytical quality assurance in veterinary drug residue analysis methods: Matrix effects determination and monitoring for sulfonamides analysis

Rodrigo Barcellos Hoff [a,b,*], Gabriel Rübensam [b], Louise Jank [a,b], Fabiano Barreto [b,c], Maria do Carmo Ruaro Peralba [a], Tânia Mara Pizzolato [a], M. Silvia Díaz-Cruz [d], Damià Barceló [d,e]

[a] Instituto de Química, UFRGS, Porto Alegre, RS, Brazil
[b] Ministério da Agricultura, Pecuária e Abastecimento, Laboratório Nacional Agropecuário—LANAGRO/RS, Porto Alegre, RS, Brazil
[c] Faculdade de Farmácia, UFRGS, Porto Alegre, RS, Brazil
[d] Department of Environmental Chemistry, IDAEA, CSIC, Jordi Girona 18-26, 08034 Barcelona, Spain
[e] Catalan Institute for Water Research (ICRA), Parc Científic i Tecnològic de la Universitat de Girona, C/Emili Grahit, 101 Edifici H2O, E-17003 Girona, Spain

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ABSTRACT

In residue analysis of veterinary drugs in foodstuff, matrix effects are one of the most critical points. This work present a discuss considering approaches used to estimate, minimize and monitoring matrix effects in bioanalytical methods. Qualitative and quantitative methods for estimation of matrix effects such as post-column infusion, slopes ratios analysis, calibration curves (mathematical and statistical analysis) and control chart monitoring are discussed using real data. Matrix effects varying in a wide range depending of the analyte and the sample preparation method: pressurized liquid extraction for liver samples show matrix effects from 15.5 to 59.2% while a ultrasound-assisted extraction provide values from 21.7 to 64.3%. The matrix influence was also evaluated: for sulfamethazine analysis, losses of signal were varying from −37 to −96% for fish and eggs, respectively. Advantages and drawbacks are also discussed considering a workflow for matrix effects assessment proposed and applied to real data from sulfonamides residues analysis.

1. Introduction

Food containing veterinary drug residues above maximum residue limit (MRL) is of major concern, since it is related directly to public health as well as international trade relationships. The demand in food regulatory control has expanded dramatically in recent decades, and residues surveillance became an important factor to be considered in international trade of commodities [1,2].

In Brazil, veterinary drug and pesticide residues analysis in animal (and also in vegetable) products are under the Ministry of Agriculture, Livestock and Supply (MAPA) management [3]. Routine analysis and methods development and validation are attributed to MAPA official laboratories network – National Agricultural Laboratories (Lanagro) – and MAPA accredited private laboratories [4]. MAPA's demand on method development and validation in residue analysis has been increased in the last decade due to the increased role of the Brazilian livestock products in national and international markets and meanly to ensure that the products traded are compliant with the safety and quality criteria required by consumers [4,5]. Wherefore, our laboratory has absorbed one important fraction of this demand in developing, validating, and submitting for accreditation methods for analysis of antimicrobial and non-antimicrobial residues in different matrices, such as milk and edible tissues of different animal species including cattle, pork, poultry, and even fish [6–10]. For these purposes, international guidelines, such as Commission Decision 2002/657/EC concerning the performance of analytical methods and the interpretation of results, and others from the US Food and Drug Administration (FDA) and the International Conference on Harmonization (ICH), are used in order to obtain methods validated according to the most stringent international criteria [11–14]. Within this issue, especial attention is paid to matrix effect (ME), which is a fundamental parameter to be determined, assessed and minimized especially when

* Corresponding author at: Ministério da Agricultura, Pecuária e Abastecimento, Laboratório Nacional Agropecuário—LANAGRO/RS, Estrada da Ponta Grossa, 3036 CEP, 91780-580 Porto Alegre, RS, Brazil. Tel.: +55 51 3248 2133; fax: +55 51 3248 2690.
E-mail address: rodrigo.hoff@agricultura.gov.br (R.B. Hoff).

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liquid chromatography–mass spectrometry (LC–MS) and/or tandem mass spectrometry (LC–MS/MS) methods are used [15–19]. The conceptualization of this phenomenon has been comprehensively reviewed by a number of authors [15,16,20,21]. Briefly, ME is related to the alteration of ionization efficiency in the ionization source by the presence of coeluting substances: the occurrence of endogenous substances originally present in the sample itself and that remains in the final extract, are appointed as the major source. A wide scope of molecules can lead to signal suppression or enhancement, especially when occurs in high concentration in the extract and elute in the same retention time window than the analyte [22]. A secondary cause are substances not originally present in the samples but able to migrate to extracts during sample preparation process as polymer and phthalates or material released by stationary phases, in bulk or in solid phase extraction (SPE) cartridges, for instance [20]. Normally, this alteration affects dramatically the method accuracy and precision and has been regarded as a critical validation item by most guidelines consulted. However, there is no consensus on how this phenomenon should be assessed during method validation. Beside, different experienced approaches of ME evaluation, based on procedures published in the scientific literature such as post-column infusion, calibration curves comparison, quantitative estimation based in standards, spiked samples and matrix-matched control comparison and control charts evaluation, has been experienced [23–27].

Although the knowledge on ME in mass spectrometry analysis has been improved in recent years, only few practical approaches has been reported for routine analysis [28–31]. In the present work, practical approaches to detect and estimate the occurrence of ME in qualitative and quantitative terms in LC–MS/MS methods for veterinary drugs residues analysis are presented and discussed. Tools for monitoring ME along the execution of routine methods are also reported. Without the purpose to exhaust the issue, the present study is proposed as a walkthrough based in relatively simple and easy techniques to be applied to analytical chemistry laboratories to deal with the critical theme of matrix effects in residues analysis.

2. Materials and methods

2.1. Analytical standards and reagents

Analytical standards with high purity ($\geq$99%) were obtained from Sigma-Aldrich (St Louis, MO, USA) namely sulfamerazine (SMR), sulfamethazine (SMZ), sulfamethoxazole (SMA), sulfamethoxypyridazine (SMPZ), sulfadiazine (SDZ), sulfapyridine (SPY), sulfadimethoxine (SDMX), sulfaguanidine (SGA), sulfacetamide (SCA), sulfabenzamide (SBZ), sulfisomidin (SIM), sulfamethizole (SMTZ), sulfaquinoxaline (SQX), sulfathiazole (STZ), sulfaisoxazole (SIX) and sulfadoxin (SDX). The metabolite N^4-acetyl-sulfamerazine (N^4-SMR) and the isotopically labelled compounds d^4-sulfamethoxazole (d^4-SMA), d^4-sulfamethazine (d^4-SMZ) and d^4-sulfadiazine (d^4-SDZ), used as surrogate and/or internal standards, were purchased from Toronto Research Chemicals (North York, Ontario, Canada).

Methanol (MeOH), acetonitrile (ACN), hexane and acetone of HPLC-grade were supplied by J. T. Baker (Deventer, The Netherlands). Diatomaceous earth was supplied by Agilent Technologies. Acetic acid and water (HPLC grade) were purchased from Merck (Darmstadt, Germany).

Individual stock standard solutions were prepared in MeOH: acetone (50:50) at 1 mg mL^{-1} and stored at −4 °C until use. Standard solutions of the mixtures of all compounds in appropriate concentrations were prepared by stock solutions dilutions using MeOH or acetone. Aliquots of each stock standard solution were diluted to obtain final concentrations of 10 μg mL^{-1} and 1 μg mL^{-1} and were stored at −20 °C.

2.2. Samples and sample preparation

Liver of different food production animals, chicken eggs, and fish muscle were obtained from Federal Inspection Service (SIF) or collected from treated animals in a farm. Liver and muscle samples were manual and finely chopped and homogenized in order to avoid slurring. Egg samples were manual and gently homogenized in order to avoid protein denaturation. After these processes, all samples were stored at −20 °C before extraction step.

Liver and fish samples were extracted by two different methods based on pressurized liquid extraction (PLE) and by ultrasounds-assisted extraction (US). A detailed discussion about these methods and validation results were submitted to publication. For PLE, an ASE 350 accelerated solvent extractor (Dionex, Sunnyvale, CA, USA) was used. Prior to extraction, d^4-SMA, d^4-SMZ and d^4-SDZ were added as surrogate standards in a concentration of 100 ng g^{-1}. Samples (0.5 g) were mixed into the PLE cells with diatomaceous earth as dispersing agent. Prior to extraction, the cells were submitted to a clean up method in order to remove lipids from the samples using hexane as solvent. PLE parameters were as follows: temperature 60 °C, 4 cycles of 5 min each one. Total flush volume of 80% and 300 s of purge with nitrogen flow were applied.

After that, the same PLE cells were submitted to a second extraction process using ACN with 0.2% acetic acid as extraction solvent. In this case, the extraction temperature was optimized at 90 °C. A preheating period of 8 min was selected and 3 cycles of 7 min each were carried out. A total flush volume of 80% and 60 s of purge with nitrogen flow were applied. Pressure was set at 1500 psi as it has been demonstrated that this parameter is not decisive in PLE.

The extracts were maintained in freezer by 1 h (at −18 °C) in order to promote protein precipitation. Following, samples were centrifuged at 1500 × *g* for 10 min in a 5810R centrifuge (Eppendorf). The supernatant was evaporated at 40 °C under nitrogen flow using a Turbo-Vap system (Zymark) until dryness. Extracts were redissolved in 1.0 mL of mobile phase mixture (water-ACN, 85:15) and transferred to a HPLC vial.

In ultrasound-assisted extraction, samples (0.5 g) were weighted in 15 mL polypropylene tubes and spiked as described for the PLE method. Following, 10 mL ACN were added and tubes were mixed in a mechanical vortex by 10 s. Afterwards all samples were placed into an ultrasonic bath for 1 h. and then stored in freezer (−18 °C) for 1 h. to promote protein precipitation. Then, samples were centrifuged at 1500 × *g* for 10 min. Supernatant was brought to dryness at 40 °C under a gentle nitrogen stream. The extracts were redissolved in 2.0 mL of the mobile phase mixture. An aliquot of 2 mL of hexane was added to remove the fat content. Afterwards, tubes were mixed in a vortex for 5 s followed by centrifugation (3500 rpm for 10 min). The lower layer was carefully transferred to a HPLC vial.

Sulfonamides analysis in eggs samples was performed as described elsewhere [32]. Briefly, samples were extracted with ACN and concentrated before reconstitution with mobile phase.

2.3. Instrumentation

LC analysis was performed with a Symbiosis™ Pico System (Spark Holland, Emmen, The Netherlands), equipped with a HPLC system consisting of an Alias™ autosampler, a loop injector and two binary pumps with a four-channel solvent selector for each one. Chromatographic separation was performed using a HPLC column Purospher® STAR (C18, ec, 150 × 4.6 mm, 5 μm) preceded by a guard column with the same packing material. The flow rate was set to 0.2 mL min^{-1}, being eluent (A) HPLC grade water acidified with 10 mM of formic acid, and eluent (B) ACN with

10 mM of formic acid. The elution gradient started with 25% of eluent (B), increasing to 80% in 10 min and to 100% in 11 min. During the next 2 min the column was kept at 100% (B), readjusted to the initial conditions in 3 min and equilibrated for 7 min. MS/MS analyses were carried out in a 4000 QTRAP hybrid triple quadrupole-linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a turbospray ionization source (ESI) working in the positive mode (ESI+).

For fish and egg analysis, the LC–MS/MS system was an Agilent 1100 series LC (Santa Clara, CA, USA) with a quaternary pump, a vacuum degasser, and an auto sampler, coupled with an API 5000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA) with an electrospray ionization source (ESI).

The optimization of the MS/MS experimental conditions was performed in previous studies and are presented in our work published elsewhere (OR: in this same issue) [33]. For increased sensitivity and selectivity, MS/MS data acquisition was performed in the selected reaction monitoring (SRM) mode, recording the two most intense transitions from the precursor ions to the product ions.

2.4. Post-column infusion method

Post-column infusions of individual standards into the MS system were performed to verify the ME of the extracts obtained for all methods, in order to verify if the whole extract or some elution fraction of the extract cause signal suppression/enhancement. This procedure was based on the experiments described by Bonfiglio et al. [23]. Briefly, blank samples extracted by the above-mentioned methods were injected into the LC–MS/MS system under the chromatographic conditions optimized for each methodology. For each injection, a standard solution of individual compound was infused into the MS system using an infusion pump, at a flow rate of 10 μL min^{-1}, through a tee-joint installed post-column. Standard solution concentration was 100 ng mL^{-1}. ME were evaluated observing signal attenuation or signal enhancement on the response of the infused analyte.

2.5. Calibration curves evaluation method

A calibration curve was prepared using standard solutions diluted in pure solvent or in mobile phase (external Standard calibration curve or "*S*"). A second calibration curve was prepared spiking a blank matrix and following with the extraction and/or cleanup procedure (Recovery calibration curve or "*R*"). Finally, a third calibration curve was made using a extract of a blank sample, which was submitted to the whole extraction and/or cleanup procedure and was spiked with standard solution at the end of the protocol, generally in the final dilution, immediately before injection (Tissue Standard calibration curve (matrix-matched) or "*TS*"). These calibration curves were prepared with the same number of points or replicates to obtain the same expected concentration in the three kinds of curve. Usually, the MRL is the central point and are constructed with a minimum of 6 point. For a MRL value of 100 μg kg^{-1} (as in the case of sulfonamides in liver), calibration curves comprehends 0, 25, 50, 100, 150 and 200 μg kg^{-1} levels. All curves were prepared and analyzed in the same batch for a more accurate comparison. After analysis, the curves were plotted and inspected visually and statistically.

Alternatively, ME was evaluated using slope ratios comparison according to the approach proposed by Romero-Gonzáles et al. [34] and Sulyok et al. [35] in a modified application of the quantitative approach of Matuszewski et al. [24]. Slopes are compared between each pair of curves obtained in the linear calibration curves prepared by spiking mobile phase (*S*), blank sample (*R*), and extract of blank sample (*TS*). Slope ratios below 0.9 or above 1.1 were associated with ion suppression and ion enhancement, respectively. For values inside that range, ME was considered negligible.

To differentiate between extraction efficiency and matrix-induced signal suppression/enhancement, the slope ratios of the linear calibration functions were calculated to yield the recovery (RE), the signal suppression/enhancement due to ME and the relative recovery, i.e. the recovery of the extraction step (RE_R) as follows:

$$RE(\%) = 100 \times slope_{spikedsamples}/slope_{liquidstandards} \quad (1)$$

$$ME(\%) = 100 \times slope_{matrix-matched\ standard}/slope_{liquidstandards} \quad (2)$$

$$RE_R(\%) = 100 \times slope_{spikedsamples}/slope_{matrix-matched\ standard} \quad (3)$$

2.6. Matrix effect quantitative estimation

The quantitative estimation of a ME, when present, was performed using a modification of the equations previously proposed by Matuszewski et al. [24]. This procedure allows determination of the ME along with the RE_R and overall "process efficiency" or method overall recovery (RE) by comparing the absolute peak areas of 3 sets of samples. Set A is composed by standard solutions (*S*). Set B is composed by samples spiked after extraction (*TS*) and set C is prepared with samples spiked before extraction (*R*). Since the values have been obtained, ME, RE_R, and RE values can be calculated as follows:

$$ME(\%) = (B/A \times 100) - 100 \quad (4)$$

$$RE_R(\%) = C/B \times 100 \quad (5)$$

$$RE(\%) = C/A \times 100 = (ME \times RE_R)/100 \quad (6)$$

2.7. Control chart

Control charts are a useful tool for the monitoring of the analytical method behavior along with-in-batch and batch-to-batch variations including those due to ME. Within this purpose, for each analysis batch, 6 quality control samples (QC) spiked at the MRL concentration level for all compounds analyzed in each method were obtained. These QC samples, as described above, are composed by 3 samples spiked after (*TS*) and 3 samples spiked before extraction (*R*). Analyte peak area of each QC sample plus standards in pure solvent are plotted in a spreadsheet using Excel software. The cells include a formula to provide average, relative standard deviation and the upper and lower limits for ME, calculated according to the control chart parameters.

3. Results and discussion

As aforementioned, there is no consensus on how ME should be evaluated during method validation, neither on the criteria that should be adopted in establish when these effects are or not occurring [36]. However, according to recent literature, two main procedures have been used to determine ME on LC–ESI–MS/MS analysis: post-column infusion, which is a dynamic technique that provides qualitative information on where ME occur along the chromatographic run; and post extraction addition, which is a static technique that quantitatively provides the ME degree at the analyte retention time [20,37]. The last technique has been preferentially used to evaluate and compare ME of different matrices in terms of relative ME. In order to evaluate the most reported approaches to ME estimation, data of two in-house developed and validated methods were used as an example.

3.1. Case study: Determination of sulfonamides (SAs) in liver, muscle and fish using two extraction procedures

Two extraction methods were developed and validated for analysis of SAs residues in liver, muscle and fish. The complete development, optimization and validation data for both methods were recently submitted to publication. Both, the PLE and the US methods were previously evaluated by their potential ME using all the described approaches.

3.2. Post-column infusion method

First, blank extracts from each extraction method were injected in a post-column apparatus for comparison with pure mobile phase in order to evaluate the variation of the standards mixture signal.

The chromatographic separation of SAs was achieved using a modification of the method published elsewhere [33,38]. Some SAs had very similar chromatographic retention time (coeluting), but were well resolved as individual peaks in the MS/MS SRM mode. The detailed optimization and discussion about the chromatography method are presented in our report published in that same issue.

To evaluate ME, first we investigated if the extraction methods could contribute with co-extractive substances that might suppress selectively the different temporal regions of the chromatogram.

As can be observed in Fig. 1, both PLE and US extracts presented signal suppression in some regions of the chromatogram. This suppression effect is more intense in the 5–6 min region. Mobile phase signal (MP, grey line) shows a standard solution infused in a post-column "*T*" connection, over a mobile phase injection. PLE (black line) and US (dashed black line) represent the signal of the respective blank extracts. The standard solution was a mixture of all analytes at a concentration level of 100 ng mL^{-1}. Any line represents a TIC signal for all monitored SRM transitions (> 36). *TS* signal is a TIC chromatogram for a blank sample spiked at 100 ng mL^{-1} injected in normal mode. The signal was multiplied by a factor of 20 times for a better comparison with the post-column infusion chromatograms. PLE and US showed very similar matrix effects over the standard signal. In the region 11–22 min both signals were virtually overlaid. In summary, analytes do not elute in the most critical suppression zones. Thus, the chromatographic conditions could be used without modifications.

3.3. Calibration curves approach

As explained before, the use of calibration curves to estimate ME can be performed in many ways. Herein, examples of each interpretation mode are demonstrated.

3.4. Graphical plot—Visual and statistical analysis

Once the 3 curves are analyzed and plotted, the following situations are considered:

Situation 1. A similar slope, non-similar intercepts. Similarity between slopes show that matrix do not interfere in the linearity of the responses. The difference between intercepts is given by the losses caused by the sample preparation process. It is expected a lower response for *R* curve. If *TS* and *S* curve could be overlapped, there is no ME. If those curves have non-similar behaviour, ME is present, but it is affecting only the signal, not the linearity. Any kind of curve can be used in this method, if an appropriate correction is applied to adjust response losses. Fig. 2 shows an example of this situation.

Situation 2. Non-similar slopes: linearity is distinct between curves. If *TS* and *R* curves had similarity in the slopes, this means that the presence of the matrix itself change the responses. In this case, only *TS* or *R* calibrations curves may be used in this method. Fig. 3 gives an example.

Situation 3. TS and *S* curves are totally overlapped. There is no ME. However, if *R* curve shows differences in intercept and slope, this means that sample preparation process change significantly the response. Thus, *R* curves may be used. See Fig. 4 for an experimental data example.

Situations 4. All curves are perfectly overlapped. No ME and recovery equals or very closely to 100%. Presumably this is just a theoretical possibility.

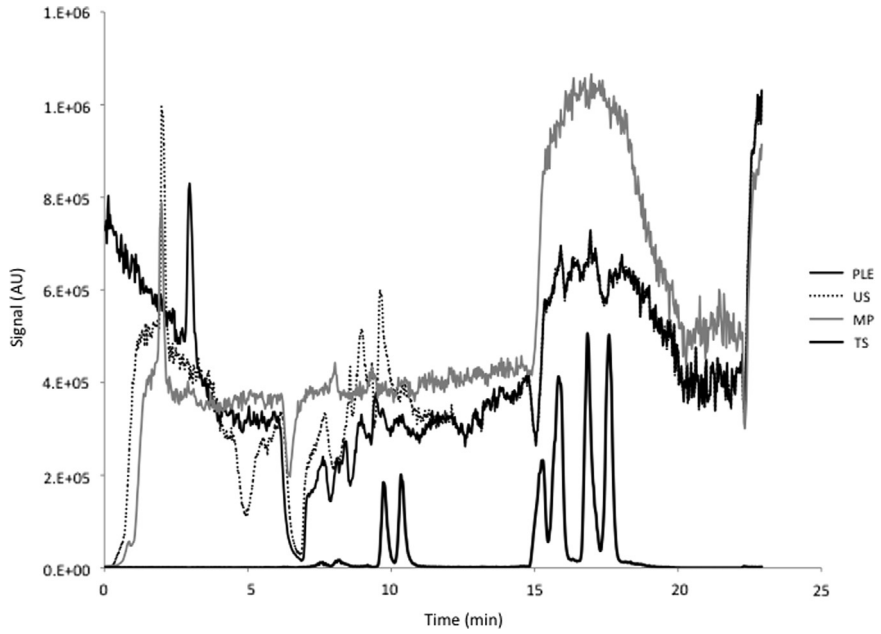

Fig. 1. Total ion chromatograms of post-column experiments. Continuous black line is a pressurized liquid extraction (PLE) blank extract; dashed black line is a ultrasound-assisted extraction (US) extract; grey line is mobile phase injection (MP) and the lower chromatogram in bold black line is a spiked tissue extract injected without post-column infusion in order to identify the elution window of the target analytes (*TS*).

In some cases, a matrix can exhibit high heterogeneity from sample to sample, which can cause significant alterations in ME [39]. This situation must be also evaluated in method development and validation. The simple superposition of plot is useful to distinguish between each calibration curve. But in some cases statistical analysis must be performed to elucidate the variation. For slope variation values, a *F*-test is applied. If $F_{cal} < F_{tab}$, the *F*-test is not significant (5% significance level), and it can be considered that the variances are similar.

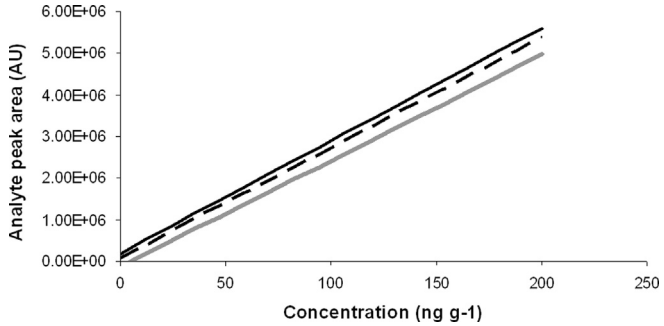

Fig. 2. Calibration curves comparison for ME evaluation: similar slopes, non-similar intercepts. Continuous line is *S* curve; dashed line is a *TS* curve and grey line represents an *R* curve.

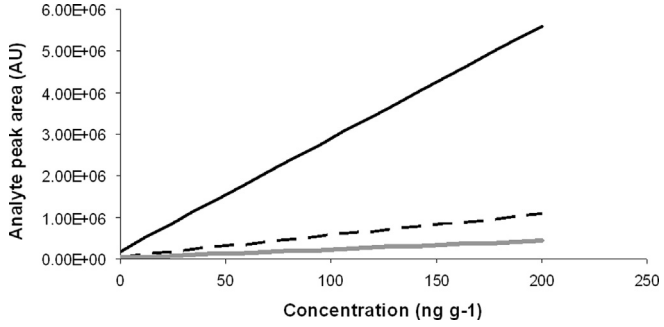

Fig. 3. Calibration curves comparison for ME evaluation: non-similar slopes. Continuous line is *S* curve; dashed line is a *TS* curve and grey line represents an *R* curve.

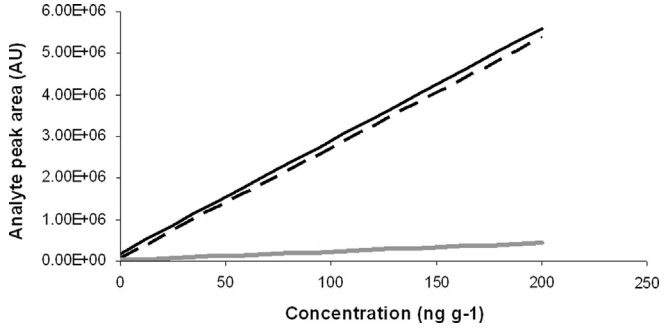

Fig. 4. Calibration curves comparison for ME evaluation: absence of ME. Continuous line is *S* curve; dashed line is a *TS* curve and dotted line represents an *R* curve.

For instance, from statistical comparison for sulfamerazine analysis in liver using matrix-matched calibration curves prepared by 2 distinct extraction methods (PLE and US) we obtained $F_{cal} < F_{tab}$ (F_{cal}=0.10 and F_{tab}=12.22 (0.05, 1, 4)) which means that the variation difference between extraction methods (PLE and US) is not significant. The slope, intercept and respective variances of both curves were calculated by the ordinary least squares method. Based on the results it was possible to conclude that PLE and US extraction methods gave equivalent responses for this analyte. In other words, matrix effects between these sample preparation methods are similar. In practical terms, it is possible to use a calibration curve prepared by US to quantify samples prepared by both methods or conversely. Similar behaviour was observed for other sulfonamides. Notwithstanding, a group of analytes was considered statistically distinct. Results are shown in Table 1.

3.5. Slope ratio and mathematical model for slopes comparison

Using Eqs. (1)–(3), quantitative values for ME and recovery were obtained (see Table 2). The slopes obtained in the calibration curve using matrix-matched samples were compared with the values obtained with standards in pure solvent. Besides the equations, slope ratio was calculated for each pair of curves to the 17 SAs included in the experiment. In this case, an acceptable range must be previously established. In the present study, a range from 0.9 to 1.1 was selected as lower and upper limits, respectively. The data were also demonstrated in Table 2.

As can be observed, using Eqs. (1)–(3), ME was very high varying from 9.4 to 73.5 % to PLE method and from 8.5 to 76.2% when US method was applied. That means an ion suppression extension as high as 91.6% in the case of SIZ, for instance. In general terms, both PLE and US methods presented very intense and highly similar ME. The use of slope ratio with acceptance limits of 0.9–1.1 shows agreement with the data obtained using the equations: an extremely intense ME for both PLE and US methods and a high degree of agreement between ME produced by PLE and US methods. When slopes of *R* and *TS* curves, some analytes showed a ratio value between tolerance range indicating no significant difference between those curves. The only analyte that showed a selective behaviour was SCA, which was the sulfonamide that suffered less effects of the matrix.

3.6. Quantitative estimation

Several degrees of ME were demonstrated, highlighting the huge variability among matrices. Depending on matrix nature, co-extractives can produce ion suppression or enhancement. For instance, Table 3 shows the quantitative ME data for some sulfonamides in fish and eggs. In the case of fish method, ME is present in the range of 30 to 40% of signal losses. RE and RE_R were in the ranges 25–41% and 46–79%, respectively. In other words, only ME can be responsible for approximately half of losses, if recovery will be considered as losses of extraction method plus

Table 1
F-test results for equality of variances between matrix-matched calibration curves prepared by PLE or US methods. F_{tab}=12.22 (0.05, 1, 4).

	SMR	SMZ	SMA	SMPZ	SDZ	SPY	SDMX	SCA	SMTZ	SQX	STZ	SIZ	SDX	N4-SMR
F_{cal}	0.10	3.76	11.42	26.79	56.73	167.26	213.88	3.04	49.27	5.03	565.65	1.51	1.17	2.06
F_{tab}	12.22	12.22	12.22	12.22	12.22	12.22	12.22	12.22	12.22	12.22	12.22	12.22	12.22	12.22
Results	$F_{cal} < F_{tab}$	$F_{cal} < F_{tab}$	$F_{cal} < F_{tab}$	$F_{cal} > F_{tab}$	$F_{cal} > F_{tab}$	$F_{cal} > F_{tab}$	$F_{cal} > F_{tab}$	$F_{cal} < F_{tab}$	$F_{cal} > F_{tab}$	$F_{cal} < F_{tab}$	$F_{cal} > F_{tab}$	$F_{cal} < F_{tab}$	$F_{cal} < F_{tab}$	$F_{cal} < F_{tab}$
Variation not significant	True	True	True	False	False	False	False	True	False	True	False	True	True	True

Table 2
Relative (RE_R) and absolute recoveries (RE), matrix effect (ME) estimated using slopes data for PLE and USE extraction methods for sulfonamides analysis in liver.

	SMR	SMZ	SMA	SMPZ	SDZ	SPY	SDMX	SGA	SCA	SBZ	SIM	SMTZ	SQX	STZ	SIZ	SDX	N4-SMR
PLE																	
RE (%)	8.8	8.1	4.7	7.6	6.0	7.8	8.3	4.2	25.2	1.5	9.2	3.0	5.0	6.7	3.0	11.7	13.9
ME (%)	23.9	25.7	13.5	28.7	16.4	24.1	29.3	12.9	73.5	6.7	30.8	19.2	20.0	11.3	9.4	34.7	24.5
RE_R (%)	36.7	31.5	35.1	26.5	36.5	32.6	28.2	32.7	34.4	23.0	29.8	15.5	25.3	59.2	31.5	33.7	56.8
USE																	
RE (%)	13.0	14.2	7.7	13.2	11.6	15.8	12.8	5.1	33.5	2.4	15.1	4.5	8.7	8.1	3.9	19.6	16.6
ME (%)	21.7	23.6	13.5	23.9	20.3	28.0	24.8	16.5	76.2	4.4	25.0	20.8	17.8	23.0	8.5	32.2	25.8
RE_R (%)	59.9	60.1	57.2	55.3	57.4	56.5	51.6	31.2	43.9	54.1	60.3	21.7	48.9	35.4	46.3	60.7	64.3
PLE slope ratio																	
TS/*S*	0.24	0.26	0.13	0.29	0.16	0.24	0.29	0.13	0.73	0.07	0.31	0.19	0.20	0.11	0.09	0.35	0.25
R/*S*	0.09	0.08	0.05	0.08	0.06	0.08	0.08	0.04	0.25	0.02	0.09	0.03	0.05	0.07	0.03	0.12	0.14
R/*TS*	0.37	0.31	0.35	0.26	0.37	0.33	0.28	0.33	0.34	0.23	0.30	0.16	0.25	0.59	0.32	0.34	0.57
USE slope ratio																	
TS/*S*	0.22	0.24	0.13	0.24	0.20	0.28	0.25	0.16	0.76	0.04	0.25	0.21	0.18	0.23	0.09	0.32	0.26
R/*S*	0.13	0.14	0.08	0.13	0.12	0.16	0.13	0.05	0.33	0.02	0.15	0.05	0.09	0.08	0.04	0.20	0.17
R/*TS*	0.60	0.60	0.57	0.55	0.57	0.56	0.52	0.31	0.44	0.54	0.60	0.22	0.49	0.35	0.46	0.61	0.64
Ratio USE/PLE																	
R/*R*	1.49	1.75	1.63	1.74	1.94	2.02	1.55	1.22	1.33	1.55	1.64	1.51	1.73	1.22	1.33	1.67	1.19
TS/*TS*	0.91	0.92	1.00	0.83	1.24	1.16	0.85	1.28	1.04	0.66	0.81	1.08	0.89	2.04	0.91	0.93	1.05

Table 3
ME quantitative estimation for sulfonamides residue analysis in fish and eggs ($n=3$ for each value).

	Sample type	Sulfonamides in fish (peak area)			Sulfonamides in eggs (peak area)		
		SMR	SMZ	SMA	STZ	SMZ	SQX
A	Standard in solvent	6.43E+05	4.09E+05	4.88E+05	7.01E+06	3.39E+06	7.56E+06
B	Matrix-matched	3.46E+05	2.56E+05	2.53E+05	2.56E+05	1.29E+05	2.49E+05
C	Spiked sample	1.59E+05	1.66E+05	2.00E+05	2.14E+05	1.22E+05	2.19E+05
	Equation						
	$ME(\%)=(B/A\times 100)-100$	−46	−37	−48	−96	−96	−97
	$RE_R(\%)=C/B\times 100$	46	65	79	84	95	88
	$RE(\%)=(ME\times R_R)/100$	25	41	41	3	4	3

ME=matrix effects; RE=Recovery; RE_R=Relative recovery.

losses by ion suppression. In the case of eggs, matrix is characterized by the intense presence of phospholipids, which are also related with a highly intense ME, causing analytes losses from 95 to 97% [40]. However, in the case of sulfonamides in eggs, recoveries were satisfactory, from 80 to 102%. The selected cases demonstrate a common profile of ME (fish) besides an extreme case (eggs), but are useful to exemplify the co-existence between intense ME and high recovery values (> 80%). The data corroborate that ME is independent of the recovery. For the eggs case, another extraction protocols should be evaluated, in order to remove the co-extractives [32].

3.7. ME continuous monitoring using control charts

The estimation of the ME during a validation procedure is mandatory, because it is used for several matrices more or less distinct those used during validation. A method for ME behavior monitoring intra-batch and inter-batch will be always necessary. Moreover, monitoring itself is not the complete task: if ME are changing, QC samples must reflect this change and procedures to assess these modifications must be available to the analyst.

Using the same kind of QC samples (*S*, *R* and *TS*) in every batch, all necessary information about the method accuracy will be available at any moment, for any batch. Accuracy data for QC samples will provide data to build a control chart to monitor ME, recovery and method accuracy.

Control charts are very useful tools to monitor analytical methods behaviour. Over time, minor and major changes were naturally occurring in routine methods. Thus, ME could be suffering changes and method adjustments will be necessary to guarantee the adequate fitness to purpose. For ME monitoring, quality control samples were inserted in each analysis batch and the results were calculated according to the quantitative ME estimation. Control charts present lower and upper limits. These limits are based on the standard deviation observed in the series. Lower and upper limits can be used as warning limits (using the average multiplied by 2 times the standard deviation), to observe trends in the results and apply preventive action before the method exceed the upper or lower limits based in 3 standard deviation values.

Table 4
Accuracy acceptability criteria.

Concentration level	Range
$\leq 1\ \mu g\ kg^{-1}$	$-50a+20\%$
$>1\ \mu g\ kg^{-1}\ a<10\ \mu g\ kg^{-1}$	$-30a+10\%$
$\geq 10\ \mu g\ kg^{-1}$	$-20a+10\%$

Acceptance criteria follow those described in 2002/657/EC Commission Decision and Brazilian analytical quality assurance guidelines [11,41]. To evaluate accuracy obtained by routine data, a critical analysis of QC samples results is performed for every batch in accordance with limits showed in Table 4.

QC samples data ($n=3$ by batch) were plotted on control charts and critically analyzed. Results for each routine analysis should be reviewed, and in case of non-compliance with criteria, it must be recorded. For a batch, if the review of QC samples shows non-

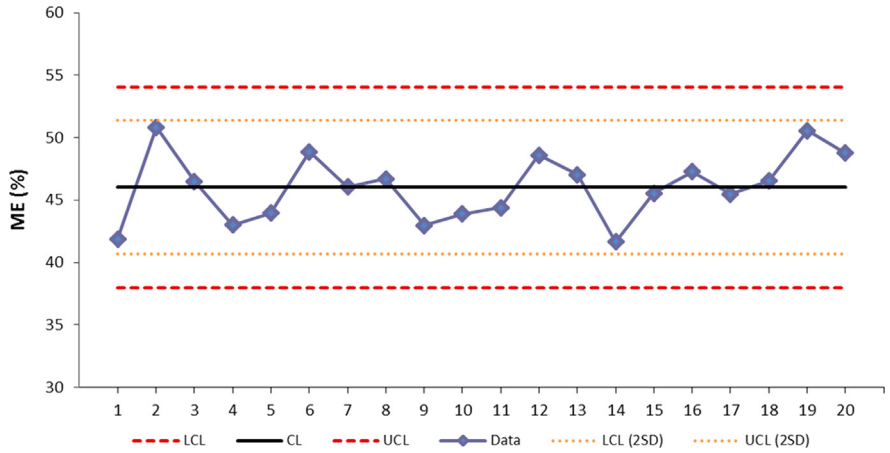

Fig. 5. Plot for matrix effects monitoring for sulfadiazine determination in liver. ME=Matrix effect; CL=central limit; UCL (2SD)=upper control limit based on 2 standard deviation; UCL=upper control limit; LCL (2SD)=lower control limit based on 2 standard deviation; LCL=lower control limit.

compliances in relation to acceptance criteria, appropriate corrective actions must be taken. Fig. 5 shows an example for ME monitoring to a sulfonamide residue analysis in liver samples. In that example, data of ME (in %) for SDZ obtained from 20 batches were plotted. The central limit (CL) was 46.4%, with an average standard deviation of 2.6%. Lower and upper control limits were obtained by adding or reducing the standard deviation value (multiplied by 2 or 3) from CL.

3.8. General remarks: Matrix effect assessment in routine methods

Concisely, in our laboratory, the following workflow is used to evaluate, minimize and monitor ME:

i. In method development, qualitative and/or quantitative approaches for ME determination can be used for analytes and surrogates/internal standards: the results can help to decide what kind of calibration curve could be used, depending on the extension of ME observed.
ii. Extraction protocols can be modified or improved to avoid co-extraction of matrix compounds: a SPE method could be more adequate than LLE, for instance. Different solvents or solvent mix can be critical to minimize ME.
iii. Once ME is observed, the post-infusion protocol is used to determine in what chromatogram region the suppression occurs.
iv. Mobile phase gradient and/or additives can be modified in order to provide analytes elution in a region with absence of suppression. Generally, just simple changes in the gradient composition are adequate to promote separation of the analytes elution window form zones with intense signal suppression. In extreme cases, solvent and even column change could be necessary.
v. Extract injection volume can be reduced in order to decrease ME, except when the analyte responses does reduce in the same extent.
vi. Evaluate the effect of surrogate/internal standards in the correction of ME.
vii. If even with these changes, ME still remain relevant, the magnitude of the effect can be monitored during routine analysis, using an accuracy control chart.

In validation data, a differentiation between recovery and ME must be clear. In mass spectrometry analysis, recovery can be deeply affected by ME although the sample preparation process showed high efficiency. As ME directly affects the yield of analytes ionization, method overall recovery have a correlation with the ME extension. In the present work, we refer to the IUPAC's recovery concept, which is the analyte yield obtained after the extraction procedure [42]. The "apparent recovery", according to IUPAC, is the degree of agreement between the nominated and calculated concentration. We use the term "relative recovery" to express the recovery value discounting the ME. Relative recovery represents the analyte losses caused only by the extraction procedure. Thus, this term should not be confused with the term "apparent recovery".

Summarizing, recovery includes losses of target compounds throughout the whole sample preparation process (extraction, concentration, derivatization, etc) plus the eventual ME. RE_R is the loss of analytes caused by the sample preparation but not include the ME.

Several approaches were considered to ME evaluation. Clearly, the methods that can be used before the method validation are more useful. The obtained data can be used to make changes or adjustments in the extraction and/or chromatography conditions to avoid or minimize the impact of ion suppression/enhancement. Once adequate conditions were established, remaining ME could be estimate using a simple approach as those based in calibration curves or QC samples. If ME is relevant in a routine method, ME could be monitored using a control chart in order to detect advance changes in method behaviour.

4. Conclusions

ME is a very frequent issue in bioanalytical methods, especially in LC–MS and LC–MS/MS based methods. Despite the fact that currently there are no established acceptable limits for ME, it is a consensus that their magnitude must be estimated and, if possible, minimized. Thus, analytes extraction procedures and/or chromatographic conditions changes could be carried out. In literature, several approaches to estimate ME were reported. Herein, we report our experience with ME estimation, minimization and continuous monitoring, applying several ME estimation strategies for analytical methods which are used in routine analysis in our laboratory. Each technique was discussed and their advantages and/or drawbacks were appointed, in order to provide a practical guide for researchers interested in assessment of ME. Based on the experience with these several techniques, the ME evaluation must be performed before the validation, using the quantitative estimation and post-column infusion, concomitantly. Using this procedure, all necessary changes and adjustments could be assessed before the validation process, improving the quality of the results and overcoming the undesirable ME.

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