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# Matrix effect marker for multianalyte analysis by LC–MS/MS in biological samples

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ABSTRACT

Matrix effects (ion suppression/enhancement) are a well-observed phenomenon in analyses of biological matrices by high-performance liquid chromatography–mass spectrometry (LC–MS). However, few simple solutions for detecting and minimizing these adverse effects have been described so far in multianalyte analysis, especially in the field of doping control.

This study describes an exhaustive characterization of matrix effects in one hundred urine samples fortified with 41 analytes (glucocorticoids and diuretics). It introduces a novel marker to identify samples in which the reliability of the results is compromised because of acute ion suppression. This new strategy strengthens the rigor of the analysis for screening purposes. Once the matrix effect is identified, a selective sample preparation is introduced to minimize the adverse ion suppression effect. That selective extraction together with the use of a deuterated internal standard permits enhancing the ruggedness of the estimation of glucocorticoid concentration in urine.



## 1. Introduction

Doping control analysis permits the detection in biological matrices, like urine or blood, of the presence of the parent compounds and/or metabolites of any of the substances included in the World Anti-Doping Agency (WADA) List of prohibited substances and methods in sport. Diuretics and glucocorticoids are two of the fifteen classes of illicit compounds or methods present on this list. Diuretics increase the urinary flow and their consumption is banned in sport for two main reasons: they can be used to dilute the urine thus masking the administration of other prohibited substances; or in sports where weight categories are involved, they can help to achieve acute weight loss (Fig. 1). Glucocorticoids (Fig. 1) are included in the prohibited list due to their anti-inflammatory properties (category S9) [1]. They are prohibited only in-competition when they are administered orally, or by intravenous, intramuscular or rectal routes. To discriminate between permitted and forbidden routes of administration, WADA recommends accredited laboratories not to report any sample with an estimated concentration of glucocorticoids or their metabolites under 30 ng mL$^{-1}$ as an adverse analytical finding [2]. It is mandatory to implement methods that permit accurate estimation of the concentration of glucocorticoids in order to report reliable results that are consistent among WADA accredited laboratories.

WADA accredited anti-doping laboratories analyze a high number of urine samples every year, for this reason, it is necessary to use generic methods of sample preparation to extract and analyze together a wide number of compounds from different therapeutic classes in order to improve time economy, laboratory productivity and reduce the volume of urine required. Frequently, laboratories use the same screening method to analyze diuretics and glucocorticoids by LC–MS/MS, due to difficulties in analyzing them by gas chromatography mass spectrometry (GC–MS).

High-performance liquid chromatography (LC) coupled with tandem mass spectrometry (MS/MS) is one of the current analytical methods of choice for doping control analysis due to the ability to analyze a wide range of doping substances in biological matrices simultaneously and with a very high level of selectivity. However, in contradiction to the common perception about LC–MS/MS based methods, the selectivity obtained with selected reaction monitoring (SRM) acquisition modes has been questioned [3]. The presence of unknown and undetectable (by SRM) components in biological matrices could induce an alteration of the analyte response that could limit and compromise the reliability of the results. This alteration of the analyte response is known as matrix effects and may be reflected in an increased (ion enhancement) or a decreased (ion suppression) signal. This complex phenomenon was first reported by Kebarle and Tang in 1993 [4] and since then, the origin, the possible mechanism, and methods to eliminate or reduce the effects have been widely discussed in relation to electrospray ionization (ESI) mass spectrometry [5–9]. These effects have also been described with the use of atmospheric pressure chemical ionization (APCI), which calls into question the mechanism of this phenomenon [10–12].

There are two main techniques to assess matrix effects: post-column infusion [10] and post-extraction addition [13]. The

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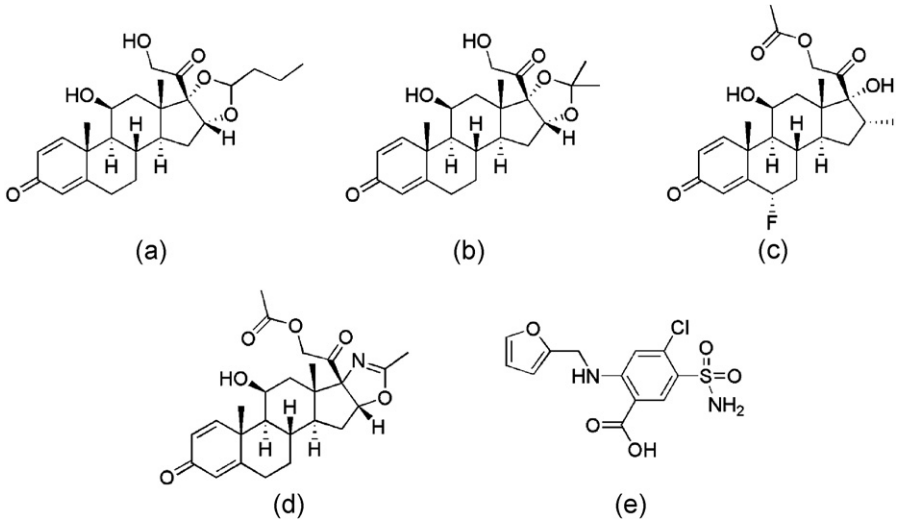


**Fig. 1.** Chemical structure of the glucocorticoids budesonide (a), desonide (b), paramethasone acetate (c), deflazacort (d) and the diuretic furosemide (e).

post-column infusion method involves continuous infusion of the analyte using a syringe pump connected via a "tee" at a point between the chromatographic column and the mass spectrometer ion source. Simultaneously, a blank extract sample is injected under the desired chromatographic conditions and the response of the analyte is monitored. This technique allows identifying the chromatographic area where the analyte will be influenced by matrix effects in a qualitative way. Every compound must be infused separately to evaluate matrix effects. This is a disadvantage if several analytes are determined in one method. Otherwise, post-extraction addition provides a quantitative assessment of matrix effects by comparison of the response of some analytes added to a post-extraction sample and the direct injection of the same amount of analytes in mobile phase. In order to quantify the matrix effect of several compounds analyzed in the same run, the post-extraction addition was chosen as the best technique.

In general, two approaches to counter matrix effects can be applied: to improve chromatographic separation or to modify the sample preparation. It is possible to adjust the chromatographic conditions to prevent the elution of the analytes in the region where ion suppression is observed. However, this generally involves increasing the chromatography times, and this can be very difficult when several compounds are analyzed simultaneously in the same run. For this reason, alternative sample extraction protocols based on different interaction mechanisms were studied.

In doping control, prevention of matrix effects is complicated due to the wide variety of endogenous and exogenous compounds that could be present in the urine. Athletes usually take medicines and dietary and nutritional supplements leading to high concentrations of the main components in the urinary sample. Many of these components present in the matrix may co-elute with doping substances present in the sample so that the response of these analytes could be affected.

To the best of our knowledge, very few papers have evaluated matrix effects on doping control in depth, although these phenomena have a serious impact on the sensitivity, accuracy and ruggedness of LC–MS/MS based methods and may lead to the non-detection of an existing analyte or underestimation of its concentration, with immediate consequences in terms of false negative reporting. In fact, in other analytical areas, these factors are taken into account. The Food and Drug Administration (FDA) recommend the identification of matrix effects during the validation process [14].

Herein we describe a simple method to characterize matrix effects in a multi-residue analysis. A novel marker to detect the presence of an acute ion suppression sample is introduced, avoiding a false negative. Additional experiments to overcome ion suppression and correctly estimate glucocorticoid concentration are proposed.

## 2. Experimental

### 2.1. Chemicals

Desoximethasone, fluorometholone, flunisolide, triamcinolone acetonide, triamcinolone, 6α-methylprednisolone, beclomethasone, betamethasone, budesonide, dichlorisone acetate, fludrocortisone acetate, flumethasone, fluocinolone, prednisolone, prednisone, altiazide and clopamide were purchased from Sigma–Aldrich (Madrid, Spain). Desonide and 1-dehydrocortexolone were purchased from Steraloids (Naxxar, Malta). Fluocortolone pivalate and 4-amino-6-(trifluoromethyl)benzene-1,3-disulphonamide (bendroflumethiazide impurity) were purchased from European Pharmacopeia (Strasbourg, France). Fluticasone propionate was purchased from British Pharmacopeia (London, United Kingdom). Paramethasone acetate, ethacrynic acid, bendroflumethizide, benzthiazide, bumetanide, chlorothiazide, hydrochlorothiazide, cyclothiazide, dichlorphenamide, hydroflumethiazide, indapamide, methyclothiazide, metolazone, polithiazide, quinethazone, trichlormethiazide and torasemide were purchased from United States Pharmacopeia (Basel, Switzerland). $D_8$-budesonide and deflazacort were purchased from Toronto Research Chemicals (Toronto, Canada). Chlortalidone, furosemide and probenecid were purchased from the World Health Organization Center for Chemical Reference Substances (Stockholm, Sweden).

Stock solutions of all compounds were individually prepared in methanol (LC grade) purchased from Scharlau (Barcelona, Spain).

The enzyme β-glucuronidase (*Escherichia coli*) was supplied by Roche Diagnostics Mannheim, Germany).

Deionized water, obtained with a Milli-Q plus apparatus Millipore (Molsheim, France) was used to prepare the mobile phase. Ammonium acetate (reagent grade) and acetonitrile (LC–MS grade) were purchased from Scharlau (Barcelona, Spain).

For the extraction procedure, formic acid (98–100%), ammonia solution 32% and *tert*-butyl methyl ether were purchased from Scharlau (Barcelona, Spain).

### 2.2. Standard solutions

Two work methanolic solutions were prepared from stock solutions, one with diuretics and the other with glucocorticoids. Both stock and working solutions were stored at temperature below −20 °C.

The pre-/post-extraction samples were fortified with 10 μL of the diuretic solution and glucocorticoid solution; 15 μL of $D_8$-budesonide (5 μg mL$^{-1}$); and 50 μL of 1-dehydro-cortexolone (2 μg mL$^{-1}$). The final concentration of each compound in the samples matched the limit of detection (LoD) shown in Table 1.

### 2.3. Sample treatment

To each test tube containing a 2.5 mL of urinary sample, 100 μL of 0.4 M $KH_2PO_4$/0.4 M $Na_2HPO_4 \cdot 2H_2O$ (pH 7) were added. The samples were hydrolyzed with β-glucoronidase (50 μL) for 1 h at 55 °C and centrifuged at 2500 rpm for 5 min. After that, the following procedures were performed to extract the free analytes:

*Procedure α*: solid-phase extraction at pH 7. Solid phase extraction using Oasis HLB 30 mg cartridges (Waters; Barcelona, Spain). The SPE cartridge was conditioned first with 0.5 mL methanol and then with 0.5 mL of Milli-Q water. The sample (2 mL) was loaded onto SPE and washed with 1 mL of Milli-Q water. Elution was carried out with 1 mL of a mixture of methanol/acetonitrile (30/70, v/v). Elution solutions were directly transferred to injection vials.

*Procedure β*: solid-phase extraction at pH 11. The pH of the sample was adjusted to 11 by adding 200 μL of 1.2 M sodium bicarbonate/1.4 M potassium carbonate. Then, a solid phase extraction was performed using Oasis HLB 30 mg cartridges (Waters, Barcelona, Spain) following procedure *α*.

*Procedure γ:* liquid phase extraction at pH 11. The pH of the sample was adjusted to 11 by adding 200 μL of 1.2 M sodium bicarbonate/1.4 M potassium carbonate Then 2 mL of *tert*-butyl methyl ether were added to the sample. The test tube was shaken for 5 min at 110 rpm and then centrifuged at 2500 rpm for a further 5 min. The organic layer was separated by freezing the aqueous layer.

*Procedure δ*: anion exchange solid-phase extraction. Solid phase extraction using Oasis MAX 30 mg cartridges (Waters, Barcelona, Spain). The SPE cartridge was conditioned with 0.5 mL methanol and 0.5 mL of Milli-Q water The sample (2 mL) was loaded onto SPE and washed with 1 mL of 5% ammonium hydroxide in Milli-Q water (v/v). Elution was carried out with 1 mL of a mixture of methanol/acetonitrile (30/70, v/v). Elution solutions were directly transferred to injection vials.

*Procedure ε*: cation exchange solid-phase extraction. Solid phase extraction using Oasis MCX 30 mg cartridges (Waters; Barcelona, Spain). The SPE cartridge was conditioned with 0.5 mL methanol and 0.5 mL of Milli-Q water. The sample (2 mL) was loaded and washed with 1 mL of 2% formic acid in Milli-Q water (v/v). Elution was carried out with 1 mL of a mixture of methanol/acetonitrile (30/70, v/v). Elution solutions were directly transferred to injection vials.

Finally, the samples were evaporated to dryness under nitrogen and then reconstituted in 400 μL of mobile phase (aqueous 1 mM ammonium acetate and 5% of acetonitrile).

Samples spiked "before extraction" indicate that the urines were spiked before any sample treatment. On the other hand, samples spiked "after extraction" indicate that the samples were spiked after they were reconstituted with mobile phase.

### 2.4. Instrumentation

Samples were analyzed using a LC 1200 (Agilent Technologies, Santa Clara, CA, USA) coupled to a triple quadrupole-linear trap mass spectrometer API 4000 (Applied Biosystem/MDS Sciex, Concord Ontario, Canada).

An automated ASPEC XL Gilson (Middletown, USA) system was used to manage solid phase extraction.

### 2.5. Chromatographic conditions

The chromatographic separation was carried out using a fused core column Poroshell 120 EC-C18 column (2.1 mm × 50 mm, overall particle size 2.7 μm, solid core 1.7 μm, Agilent Technologies) and solvents were 1 mM ammonium acetate (A) and acetonitrile (B). The flow was set to 350 μL min$^{-1}$ and column oven to 30 °C. The gradient elution was conducted starting at 80% A for 0.6 min, at 0.75 min A was decreased to 2% and maintained for a further 2.6 min followed by re-equilibration at 80% A. The total run time was 7.5 min.

The mobile phase A (1 mM ammonium acetate) was prepared daily.

### 2.6. Mass spectrometer conditions

The MS was equipped with a TurboIon Spray source (electrospray ionization) operating in negative ionization mode. The ion spray voltage was set at −4500 V. Nebulizer, collision and curtain gases were $N_2$. The pressures were set at 50 psi for the nebulizer and for the drying gases and at 10 psi for the curtain gas. Source temperature was fixed at 350 °C.

Collision energy, declustering potential and others acquisition parameters were optimized by 10 μL min$^{-1}$ infusion of 10 μg mL$^{-1}$ standard solutions in mobile phase. The final conditions and the retention time of each compound are shown in Table 1.

Data acquisition, data handling and instrument control were performed with Analyst™ software version 1.5 (Applied Biosystems). This software included the Schedule MRM Algorithm™ tool that generates small dynamic periods or segments of acquisition around the expected retention time (detection window) of the analyte of interest. The length of the segments and the total scan time (cycle time) can be fixed by the user (in this particular case the detection window was fixed at 45 s and the cycle time at 0.5 s). The algorithm optimizes the dwell time based on the number of transitions that are co-eluting. In this way, the highest sensitivity, accuracy and reproducibility of the chromatographic signals are obtained. However, if the expected retention time is fixed at 0 min for one substance, that compound will be monitored during all the chromatographic time. This option could be very useful for detecting analytes in which retention time moves due to matrix effects.

### 2.7. Assessing matrix effects

Under the accreditation ISO 17025, the laboratory implemented an LC–ESI-MS/MS method to monitor 20 glucocorticoids and 21 diuretics in urine samples. At least two transitions were monitored for each compound. The use of ESI in negative mode permitted analyzing diuretics and glucocorticoids in a simple run of 7.5 min. Otherwise, the negative mode is usually considered as more specific [15,16]. The detection limits achieved with this method are shown in Table 1.

To evaluate the matrix effects, blank urines were extracted with the routine screening method (procedure *α*) and spiked with pure standards of all tested compounds. The response of the analyte was compared to a neat standard solution. The matrix effect (ME) value for each sample was calculated as follows [17]:

$$\mathrm{ME}\,(\%) = \frac{B - A}{A} \times 100,$$

**Table 1**
Chromatographic and spectrometric parameters and characterization of matrix effects of analyte targets in urinary samples ($N = 100$).

| Compound | $R_t$ (min) | Precursor ion (DP, V) | Product ion (CE, eV) | LoD (ng mL$^{-1}$) | Average value of matrix effects (%) | Number of samples with ion suppression values >25% |
|---|---|---|---|---|---|---|
| **Diuretics** | | | | | | |
| Altiazide | 3.0 | 382 (−41) | 341 (−24); 269 (−40) | 50 | 11 | 12 |
| Bendroflumethiazide impurity | 0.9 | 318 (−96) | 214 (−34); 78 (−62) | 25 | −16 | 32 |
| Bendroflumethizide | 3.2 | 420 (−46) | 289 (−34); 197 (−68) | 25 | 14 | 5 |
| Benzthiazide | 2.9 | 430 (−61) | 308 (−32); 228 (−60) | 25 | −37 | 70 |
| Cyclothiazide | 3.2 | 388 (−101) | 269 (−40); 205 (−46) | 100 | 37 | 8 |
| Clopamide | 2.6 | 344 (−86) | 78 (−61); 167 (−34) | 50 | −20 | 50 |
| Chlorothiazide | 0.5 | 294 (−56) | 179 (−66); 115 (−78) | 50 | −84 | 99 |
| Chlortalidone | 1.4 | 337 (−52) | 146 (−29); 190 (−23) | 50 | 0 | 6 |
| Metolazone | 3.0 | 364 (−91) | 257 (−30); 231 (−32) | 25 | −23 | 44 |
| Torasemide | 2.8 | 347 (−31) | 262 (−20); 196 (−42) | 25 | 17 | 8 |
| Dichlorphenamide | 1.4 | 303 (−91) | 267 (−24); 224 (−28) | 250 | 20 | 6 |
| Ethacrynic acid | 2.8 | 301 (−16) | 243 (−20); 207 (−38) | 50 | −23 | 40 |
| Indapamide | 3.1 | 364 (−70) | 189 (−36); 132 (−34) | 50 | 6 | 6 |
| Bumetanide | 2.8 | 363 (−75) | 80 (−44); 238 (−24) | 50 | −22 | 43 |
| Polithiazide | 3.2 | 438 (−51) | 398 (−24); 324 (−28) | 25 | 20 | 4 |
| Quinethazone | 0.7 | 288 (−56) | 245 (−30); 209 (−28) | 250 | −34 | 66 |
| Furosemide | 1.1 | 329 (−41) | 285 (−22); 205 (−30) | 100 | 3 | 7 |
| Hydrochlorothiazide | 0.7 | 296 (−41) | 269 (−26); 126 (−48) | 50 | −37 | 76 |
| Hydroflumethiazide | 1.0 | 330 (−26) | 303 (−32); 160 (−52) | 50 | 2 | 4 |
| Methyclothiazide | 2.9 | 358 (−41) | 322 (−20); 194 (−34) | 50 | −28 | 59 |
| Probenecid | 2.8 | 284 (−41) | 240 (−20); 164 (−30) 140 (−36) | 10 | −29 | 58 |
| **Glucocorticoids** | | | | | | |
| Paramethasone acetate | 3.3 | 493 (−21) | 361 (−34); 373 (−26) | 30 | −2 | 11 |
| Fluticasone propionate | 3.7 | 559 (−46) | 413 (−28); 329 (−28) | 30 | 13 | 2 |
| Prednisone | 2.9 | 417 (−26) | 327 (−28); 299 (−26) 149 (−60) | 30 | −19 | 39 |
| 6α-methylprednisolone | 3.0 | 433 (−41) | 309 (−46); 294 (−54) | 30 | −33 | 67 |
| Fluocortolone pivalate | 4.0 | 519 (−1) | 343 (−34); 323 (−38) | 30 | 10 | 2 |
| Triamcinolone acetonide | 3.1 | 493 (−31) | 337 (−34); 375 (−20) | 30 | −13 | 27 |
| Flunisolide | 3.1 | 493 (−36) | 185 (−40); 375 (−20) | 30 | −1 | 11 |
| Triamcinolone | 1.7 | 453 (−26) | 345 (−38); 363 (−20) | 30 | −8 | 5 |
| Fluocinolone | 3.1 | 511 (−31) | 431 (−28); 355 (−36) | 30 | 0 | 18 |
| Dichlorisone acetate | 3.5 | 513 (−21) | 417 (−14); 453 (−12) | 30 | 13 | 1 |
| Prednisolone | 2.9 | 419 (−41) | 295 (−48); 280 (−48) 187 (−44) | 30 | −22 | 47 |
| Fludrocortisone acetate | 3.2 | 481 (−30) | 421 (−14); 349 (−24) | 30 | 20 | 7 |
| Betamethasone | 3.0 | 451 (−21) | 307 (−50); 292 (−54) | 30 | −25 | 59 |
| Desonide | 3.1 | 475 (−30) | 357 (−16); 339 (−22) | 30 | −10 | 43 |
| Budesonide | 3.3 | 489 (−31) | 357 (−24); 339 (−24) | 30 | 16 | 5 |
| Beclomethasone | 3.1 | 467 (−46) | 377 (−20); 341 (−30) | 30 | −19 | 37 |
| Desoximetasone | 3.2 | 435 (−36) | 121 (−40); 355 (−22) | 30 | 17 | 7 |
| Fluorometholone | 3.2 | 435 (−31) | 255 (−32); 355 (−22) | 30 | −3 | 14 |
| Flumethasone | 3.0 | 469 (−41) | 379 (−28); 325 (−42) | 30 | 1 | 17 |
| Deflazacort | 3.2 | 500 (−41) | 339 (−36); 398 (−26) | 30 | −13 | 24 |
| **Internal standards** | | | | | | |
| 1-Dehydro-cortexolone | 3.0 | 403 (−26) | 313 (−28) | 40 | −33 | 62 |
| $D_8$-budesonide | 3.3 | 497 (−50) | 339 (−24) | 30 | 12 | 4 |

where $A$ is the analyte peak area in mobile phase and $B$ is the analyte peak area for urine spiked after extraction.

The response of the analyte in mobile phase provides 0% of the matrix effect, the ideal situation. When the value of the matrix effect is negative, it indicates that the response of an analyte in the urine is lesser than in the mobile phase, so the urine presents an ion suppression effect. On the contrary, if matrix effects are positive, the urine presents an ion enhancement effect. Process efficiency (PE) and recovery ($R$) were also calculated:

$$\mathrm{PE}(\%) = \frac{C}{A} \times F \times 100$$

$$R(\%) = \frac{C}{B} \times F \times 100,$$

where $C$ is the analyte peak area for urine spiked before extraction and $F$ is the correction factor to adjust volume variations.

Process efficiency represents the combination of matrix effects and the recovery of the analyte from the matrix by the sample extraction process. Therefore, a large effect on ion suppression means low process efficiency values. Nevertheless, if the recovery is high enough, ion suppression could be compensated and analytes could be detected without problems.

## 3. Results and discussion

### 3.1. Characterization of matrix effects: prevalence and consequences of acute ion suppression

To start the study one hundred samples previously reported as negative, were randomly selected. In order to evaluate the prevalence and the degree of matrix effects that could affect each compound, a descriptive study of matrix effects on the detection of diuretics and glucocorticoids by LC–ESI-MS/MS was carried out. Results are presented in Table 1.

These results show the complexity of the problem as all the compounds evaluated are affected by matrix effects. Moreover, these effects are observed in all the samples. In the case of diuretics, their detection was not compromised. Even in the most acute case, chlorothiazide, 99% of the samples, had ion suppression values

**Table 2**
Detection of several glucocorticoids in samples with ion suppression values greater than 40–60%.

| Glucocorticoid | Matrix effect | Process efficiency | Recovery | *S/N* |
|---|---|---|---|---|
| Fluorometholone | −57 | 38 | 89 | 29 |
| Flunisolide | −58 | 40 | 98 | 57 |
| Triamcinolone acetate | −56 | 39 | 89 | 233 |
| 6α-Methylprednisolone | −62 | 27 | 71 | 275 |
| Beclomethasone | −53 | 49 | 103 | 11 |
| Betamethasone | −66 | 28 | 83 | 141 |
| Desonide | −47 | 49 | 92 | 86 |
| Flumethasone | −53 | 39 | 83 | 704 |
| Fluocinolone | −60 | 33 | 83 | 305 |
| Prednisolone | −57 | 34 | 79 | 154 |
| Prednisone | −55 | 39 | 87 | 163 |
| Deflazacort | −51 | 45 | 91 | 12 |

higher than 25% (the average value of matrix effects was −84%) and average signal-to-ratio was around 300.

Moreover, WADA establishes a minimum required performance level (MRPL) of 250 ng mL$^{-1}$ for this category [2] and, in most of the cases, the detection limit reached by LC–MS/MS is much lower. It is noteworthy that in 3% of the cases the retention time of furosemide shifted 0.5 min with respect to the rest of the samples. This could be interpreted as another consequence of matrix effects. The shifting was not observed for the rest of the compounds. Software tools such as Schedule MRM Algorithm used to improve the quality of an instrumental analysis method could in fact prevent the consistent detection of furosemide. The problem arises because furosemide was only monitored in the range $1.1 \pm 0.38$ min (where 1.1 min is the retention time of furosemide in most of the samples). Fortunately, it was possible to overcome the problem by fixing the retention time at 0 min, which allows to be monitors for the whole analysis the compound would be monitored during the whole run (Fig. 2). For this reason, when tools like Schedule MRM Algorithm™ are used, for certain chromatographic conditions, it is worth controlling the matrix effects to avoid losing compounds.

In the case of glucocorticoids, the scenario is completely different, because the concentration must be estimated in order to discriminate if the glucocorticoid comes from a forbidden route of administration or not. Moreover, according to WADA TD2010MRPL [2], the detection limit of the laboratory for such compounds should be at least 30 ng mL$^{-1}$, much lower than that for diuretics The shift of retention time was not observed for the glucocorticoids; however, ion suppression became a serious problem that affected not only the detection but also the quantification.

The ion suppression effect has been described previously for some prohibited substances in anti-doping research [18–21]. According to some publications, when ion suppression values are lower than 25% [19] the results are considered acceptable. In general terms, for glucocorticoids, even with higher ion suppression, due to the elevated detection capability of the method, the detection is not in danger [20].

We found that more than 90% of the tested samples presented ion suppression lower than 25%. However, for compounds shown in Table 2, the impact was greater. A representative sample for each glucocorticoid was studied in detail to prove that ion suppression higher than 40–60% was not an impediment for detecting the substance; however, it could be a real problem for an appropriate quantitation matrix effect (ME), process efficiency (PE) and recovery (*R*) were calculated for comparison purposes.

According to the signal-to-noise ratio presented in Table 2, and despite the high ion suppression effects, on these samples the glucocorticoids could be detected correctly.

Among all the samples, there were four cases where the ion suppression effect was much more pronounced for almost all the glucocorticoids. These urines contained unknown compounds that caused a reduction of the signal area by more than 70% for most of the glucocorticoids. In these extreme cases, the ion suppression could compromise analyte detection. Some of the glucocorticoids had very weak signals when the four identified urines were spiked at a concentration of 30 ng mL$^{-1}$ before the extraction. Deflazacort and budesonide could not be detected at all in these spiked urines.

In fact, when all those urines were spiked with glucocorticoids before the extraction, some compounds were detected with a really weak signal and with regard to a few glucocorticoids, deflazacort and budesonide, it was not possible to detect them. This is extremely important because it means that 4% of the samples analyzed in a doping-control laboratory could have an ion suppression effect that could lead to a false negative. It is noteworthy that three of these samples were the same ones in which the furosemide shifted 0.5 min with respect to the rest of the samples in the diuretic analysis. It proves that the retention time shift of furosemide was due to matrix effect. Fig. 3 shows the matrix effect observed in one of the samples with acute ion suppression compared with a more common situation. Ten replicates of each of the two samples were extracted. As can be seen, the matrix effect of both samples was completely different. The marked ion suppression of one of them could jeopardize the detection of some glucocorticoids.

To the best of our knowledge, simple solutions for detecting acute ion suppression have not been described before. An efficient methodology would be the use of a compound that permits identification of cases with extreme ion suppression, and then, special measures to address the problem of all those samples could be activated to avoid problems of non-detection or wrong quantification.

### 3.2. Marker of ion suppression effect

For the whole group of compounds tested, it was observed that the glucocorticoid budesonide showed an interesting matrix effects behavior. Ninety-six percent of the samples included in the study presented a loss of sensitivity for budesonide lower than 30% due to ion suppression, and only 4% of the samples lost more than 50% of sensitivity through matrix effects. At the same time, there were no samples with a matrix effect on budesonide between −30 and −50%. In this sense, budesonide seemed to be very suitable as an indicator of samples with severe matrix effects; in fact, it allowed identification of the four samples that presented acute ion suppression for almost all the glucocorticoids (Fig. 3). Obviously, budesonide cannot be used because it is included in the WADA list of forbidden substances. However, the use of an isotopically labeled (SIL) analog like $D_8$-budesonide, could be of great utility. To verify the suitability of $D_8$-budesonide, the ion suppression of this compound was calculated for the one hundred urine samples. The results are shown in Fig. 4. Ninety-six percent of the samples had an ion suppression value lower than 20% and only 4% of the samples presented a matrix effect below −90%. There are no samples with ion suppression values between 17 and 91%, so there is a wide range to discriminate samples with acute ion suppression (Fig. 4).

In order to identify a cut-off value for the marker, a normal tolerance one-sided interval was constructed. The results obtained from the mean value and the standard deviation of the $D_8$-budesonide matrix effects in all the samples tested ($N = 100$), are shown in Fig. 5. As can be seen, with a 95% level of confidence, 99% of the samples presented matrix effects in $D_8$-budesonide above −58%. Therefore, samples where $D_8$-budesonide presents matrix effects below −58% could be indicating that the sample shows acute ion suppression. In consequence, the analysis of that sample could lead to a false negative.

For a reliable marker, it is important that the values of matrix effects of the marker are consistent with those of other glucocorticoids. As indicated in Fig. 6, in those samples where $D_8$-budesonide

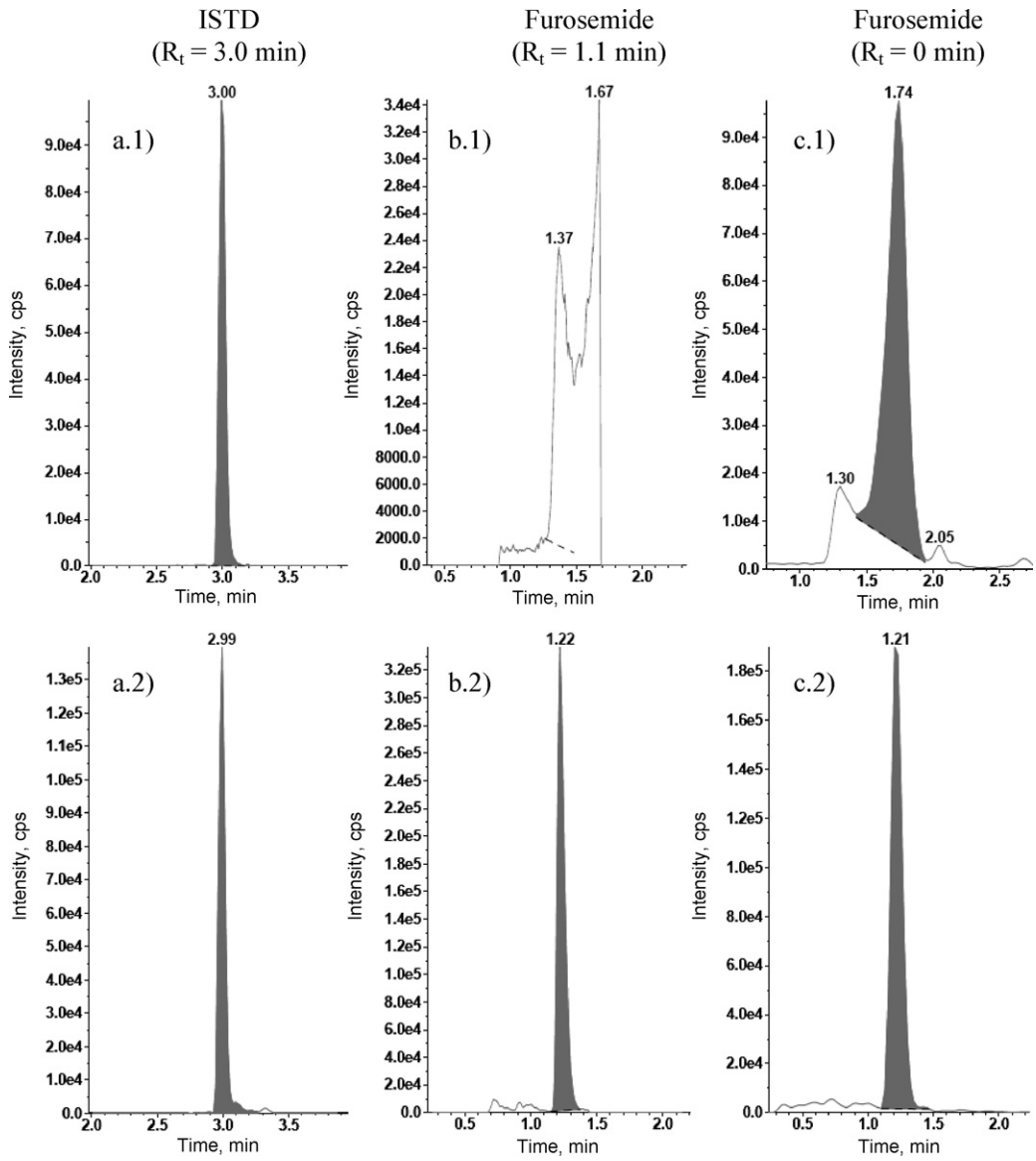


**Fig. 2.** Reconstructed ion chromatogram for the detection of the internal standard and the diuretic furosemide in two samples: (1) a sample where furosemide retention time was shifted compared to a more common situation and (2) a sample the behavior of which corresponds to a normal situation. (a) Internal standard (1-dehydro-cortexolone) chromatogram acquired fixing a retention time of 3.0 min. (b) Furosemide chromatogram acquired fixing a retention time of 1.1 min. (c) Furosemide chromatogram acquired for all the chromatographic time.

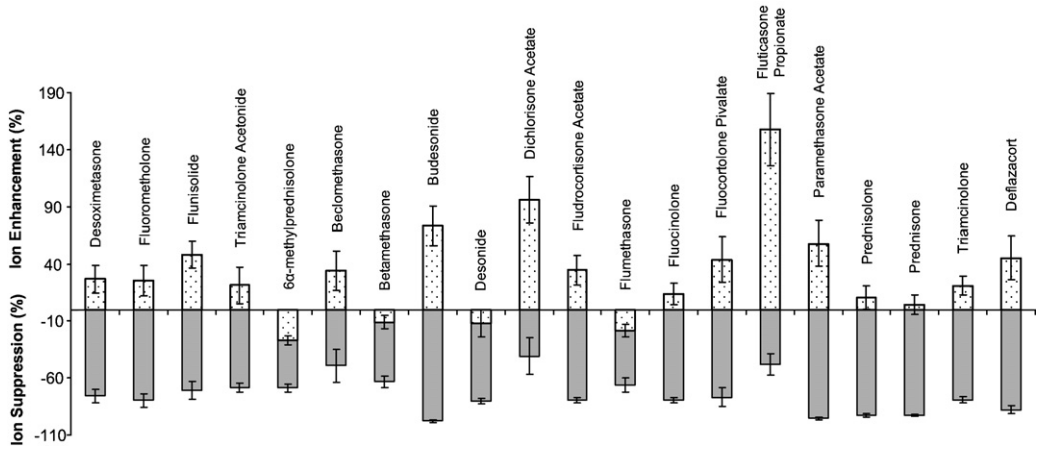


**Fig. 3.** The matrix effect was observed in two urine samples ($N = 10$) spiked with several glucocorticoids. In dotted white the common sample and in dotted gray the sample with acute ion suppression effect.

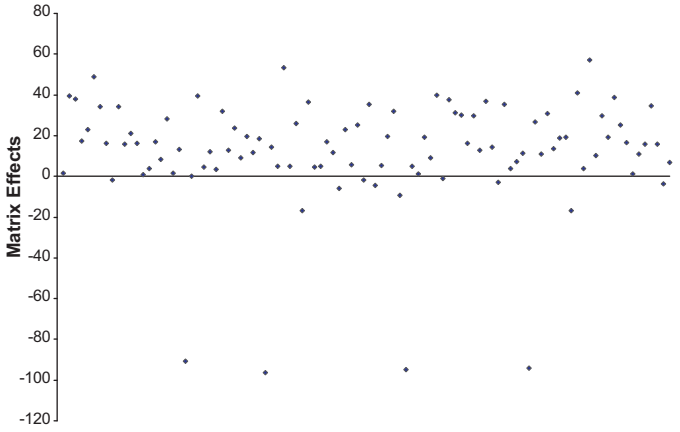


**Fig. 4.** Matrix effect observed in one hundred samples relative to the glucocorticoid $D_8$-budesonide.

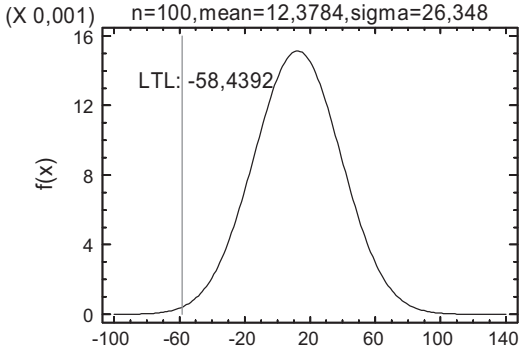


**Fig. 5.** A lower one-sided tolerance interval for matrix effects.

showed ion suppression higher than 80%, several glucocorticoids also presented high ion suppression values. On the other hand, samples where $D_8$-budesonide presented mostly positive values of matrix effects, glucocorticoids were safely detected (the mean value of the matrix effect is rarely lower than −20%). The variability of matrix effects was very high because of the differences in urinary matrices. Nevertheless, there was a great difference between the two groups of urines. The variability of matrix effects in both groups rarely overlapped, and when this happened, the detection of these glucocorticoids was usually not compromised in any case (Table 1). The detection of glucocorticoids in samples where $D_8$-budesonide did not indicate alarming values of ion suppression was reliable. Only a few samples of this group with ion suppression higher than 60% in 6α-methylprednisolone and betamethasone were detected. The signal-to-noise ratio for these two glucocorticoids in the samples with the lowest matrix effect was greater than 3 (6α-methylprednisolone, matrix effect −64%, $S/N = 235$; betamethasone, matrix effect −66%, $S/N = 72$) so the reliability of the analysis was guaranteed. These results demonstrate that $D_8$-budesonide acts as a good indicator of samples in which suppression can lead to a false negative.

### 3.3. Strategies to overcome ion suppression effects

Different extraction procedures were tested in order to address the problems of acute ion suppression: solid-phase extraction at pH 7 ($\alpha$), solid-phase extraction at pH 11 ($\beta$), liquid–liquid extraction at pH 11 ($\gamma$), and anion ($\delta$) and cation ($\varepsilon$) exchange solid-phase extraction. These sample preparations were described in detail in Section 3.3.

Matrix effect, process efficiency and recovery were measured to compare different extraction methods in the four samples that presented acute ion suppression. Table 3 shows the results obtained for four glucocorticoids in those samples. The glucocorticoids were selected based on their different chromatographic behavior and matrix effect degree.

Anion exchange solid-phase extraction (protocol $\delta$) significantly reduced ion suppression effects while the recovery of the compounds was greater so the process efficiency also increased. This extraction method would detect glucocorticoids regardless of matrix effects; however, due to the acid character of diuretics, they were not extracted efficiently. For this reason, anion exchange solid-phase extraction cannot be used as a screening method, but it can be used for additional experiments in cases of acute ion suppression.

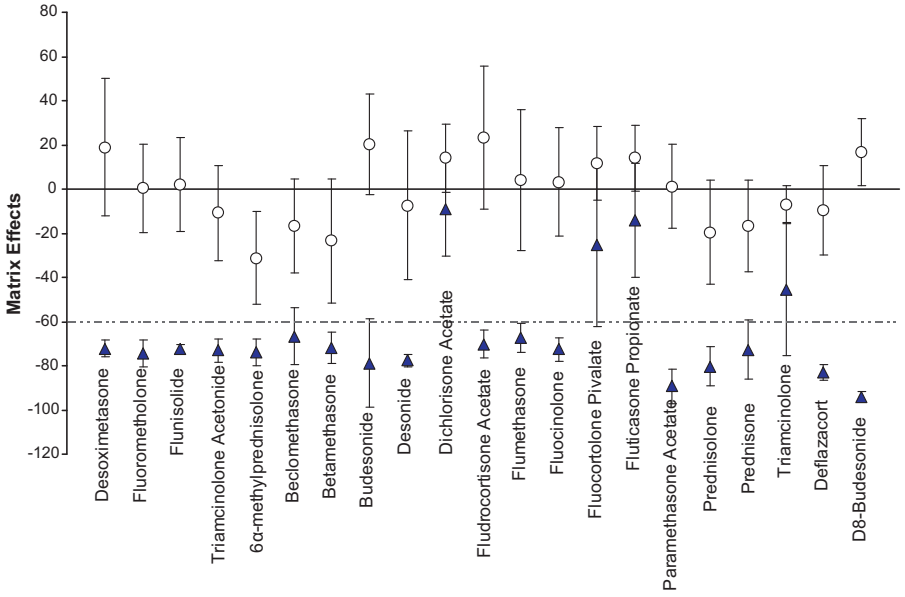


**Fig. 6.** Matrix effects in two groups of samples: samples with acute ion suppression, $N = 4$ (▲) and the rest of samples, $N = 96$ (○).

**Table 3**
Matrix effect (ME), extraction recovery (R) and process efficiency (PE) measured according the sample preparation procedure for a selection of four glucocorticoids in samples with acute ion suppression ($N = 4$).

| Extraction procedure | α: Hydrophobic solid phase extraction pH7 | | | β: Hydrophobic solid phase extraction pH11 | | | γ: Liquid phase extraction pH 11 | | | δ: Anion exchange solid phase extraction | | | ε. Cation exchange solid phase extraction | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ME | R | PE | ME | R | PE | ME | R | PE | ME | R | PE | ME | R | PE |
| Flunisolide | −63 | 71 | 19 | −85 | 94 | 12 | −57 | 120 | 49 | 12 | 84 | 95 | −67 | 90 | 26 |
| 6α-Methylprednisolone | −70 | 72 | 21 | −78 | 67 | 15 | −71 | 91 | 27 | −42 | 70 | 41 | −35 | 82 | 48 |
| Fluticasone propionate | −2 | 28 | 27 | 2 | 67 | 68 | −7 | 18 | 17 | 33 | 43 | 57 | −14 | 18 | 11 |
| Paramethasone acetate | −55 | 105 | 40 | −33 | 71 | 49 | −17 | 67 | 54 | 2 | 81 | 81 | −62 | 117 | 43 |

### 3.4. Estimation of the concentration of glucocorticoids

Once the detection is assured, the last factor needing to be controlled is the estimation of the glucocorticoids concentration. Although anion exchange solid-phase extraction reduces matrix effects, an internal standard is still necessary to correct any variation to improve quantitative analysis. The internal standard and the analyte have to co-elute to ensure the ionization under the same conditions. Therefore, a stable isotopically labeled (SIL) analog is commonly used as the internal standard. Because some publications [22,23] indicate that the utilization of an SIL does not guarantee a successful analysis, in the present study three types of internal standards were tested (ISTDs): a stable isotopically labeled (SIL) analog, a structural analog compound [24] or a compound with the same retention time although it is structurally different. In order to select the best pattern of quantification, only the glucocorticoid budesonide was studied. Furthermore, budesonide is the glucocorticoid that most often gives rise to adverse analytical findings [25]. $D_8$-budesonide was selected as the isotopically labeled (SIL) analog, in which eight deuterium atoms in the alkyl chain of the acetonide protecting group differ from budesonide. Desonide was tested as the structural analog compound, only the acetonide protecting group differs from budesonide resulting in a 0.2 min difference in retention time (Fig. 1). Finally, paramethasone acetate was also tested because it presents the same retention time although its structure includes additional functional groups and alkyl chains that make it structurally different from budesonide (Fig. 1). Although desonide and paramethasone acetate are other glucocorticoids included in the WADA prohibited list they were only selected to study a suitable internal standard pattern.

Ten negative urine samples of the one hundred used for the study of ion suppression were fortified with budesonide and the ISTDs at 30 ng mL$^{-1}$ before the sample preparation following the extraction procedure $\delta$ for five days. The four urines that presented acute ion suppression were included among those samples.

The analyte/ISTD response ratio ($Q$) was calculated based on the budesonide and ISTD peak area in every sample. A randomly selected sample was considered as the reference so the value of 30 ng mL$^{-1}$ was assigned for the concentration. The concentration of the rest of the samples was determined comparing the $Q_{sample}$ and the $Q_{reference}$ as follows:

$$\text{Concentration sample (ng mL}^{-1}) = \frac{Q_{sample}}{Q_{reference}} \times 30 \text{ ng mL}^{-1}$$

Table 4 shows the estimation of the budesonide concentration in every sample using different internal standards (mean of $N = 5$).

The best results were obtained when $D_8$-budesonide was used as the internal standard. Independently of the matrix effect of the samples, the estimated concentration was very close to 30 ng mL$^{-1}$ and the variability among samples was low. In this case, the analyte/ISTD response ratio remained constant with matrix effects changes. However, when desonide was used, the estimated concentration was higher than 30 ng mL$^{-1}$ and the variability among samples was very high as well. In fact, in one case the estimated concentration was more than twice the expected value. This unreasonable value was observed just in the sample that presented the most acute ion suppression. Although the extraction procedure had reduced the ion suppression, this effect was still present and especially marked in this sample, affecting desonide more than budesonide. When paramethasone acetate was used as the internal standard, the estimated budesonide concentration was lower than 30 ng mL$^{-1}$ and the variability among samples was again very high. In conclusion, in this case, the use of $D_8$-budesonide corrected almost all matrix effects and permitted the correct estimation of the budesonide concentration independently of the urine sample. However, a stable isotopically labeled (SIL) analog was required to quantify each of the other glucocorticoids.

**Table 4**
Estimation of the budesonide concentration in nine urines, for five days, using three different internal standards. Samples with acute ion suppression effect are indicated as (*).

| | $D_8$-budesonide | Desonide | Paramethasone acetate |
|---|---|---|---|
| (*) | 30.67 | 34.08 | 10.56 |
| | 31.76 | 32.67 | 9.31 |
| (*) | 31.09 | 42.49 | 8.34 |
| (*) | 31.08 | 87.72 | 7.99 |
| (*) | 31.72 | 36.94 | 7.81 |
| | 32.10 | 33.38 | 8.40 |
| | 33.13 | 32.60 | 20.34 |
| | 30.59 | 33.02 | 9.22 |
| | 30.67 | 31.81 | 32.46 |
| Mean | 31.42 | 40.52 | 12.71 |
| DS | 0.84 | 18.00 | 8.37 |
| CV | 3% | 44% | 66% |

## 4. Conclusions

Although LC–MS/MS is a highly selective and sensitive technique, it is not completely free from adverse aspects and limitations like the response-reducing phenomenon of ion suppression. Matrix effects appear to be almost impossible to eliminate but it is possible to overcome the major threats. Around 4% of the samples analyzed in a doping-control laboratory exhibit acute ion suppression effects that could lead to a false negative. A novel marker criterion can be used to alert the analyst to the presence of these kinds of samples. $D_8$-budesonide solves that function correctly. When in a sample the matrix effects of $D_8$-budesonide are lower than −58%, it indicates that the sample presents acute ion suppression and therefore the glucocorticoids analysis is compromised.

Two important considerations must be taken into account with samples detected by the marker, on one hand the retention time of furosemide was able to shift significantly. The use of Schedule MRM Algorithm™ requires a previous evaluation of matrix effects in order to optimize the segment range of acquisition or the necessity to acquire a signal in all the chromatogram. On the other hand, glucocorticoids must to be extracted following an anion exchange solid-phase extraction to ensure the detection of all of them. If the glucocorticoid budesonide is present in a sample, its

concentration can be estimated correctly using $D_8$-budesonide as the internal standard. Other stable isotopically labeled (SIL) analogs are required to quantify the rest of the glucocorticoids.

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