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# Systematic evaluation of matrix effects in supercritical fluid chromatography *versus* liquid chromatography coupled to mass spectrometry for biological samples

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ABSTRACT

Matrix effects (ME) is acknowledged as being one of the major drawbacks of quantitative bioanalytical methods, involving the use of liquid chromatography coupled to mass spectrometry (LC-MS). In the present study, the incidence of ME in SFC-MS/MS and LC-MS/MS in the positive mode electrospray ionization (ESI+) was systematically compared for the analysis of urine and plasma samples using two representative sets of 40 doping agents and 38 pharmaceutical compounds, respectively. Three different SFC stationary phase chemistries were employed, to highlight the importance of the column in terms of selectivity. Biological samples were prepared using two different sample treatments, including a non-selective sample clean-up procedure (dilute and shoot (DS) and protein precipitation (PP) for urine and plasma samples, respectively) and a selective sample preparation, namely solid phase extraction (SPE) for both matrices.

The lower susceptibility to ME in SFC *vs.* reversed phase LC (RPLC) was verified in all the experiments performed on urine, and especially when a simple DS procedure was applied. Also, with the latter, the performance strongly varied according to the selected SFC stationary phase, whereas the results were quite similar with the three SFC columns, in the case of SPE clean-up. The same trend was observed with plasma samples. Indeed, with the PP procedure, the occurrence of ME was different on the three SFC columns, and only the 2-picolylamine stationary phase chemistry displayed lower incidence of ME compared to LC-MS/MS. On the contrary, when a SPE clean-up was carried out, the results were similar to the urine samples, with higher performance of SFC *vs.* LC and limited discrepancies between the three SFC columns. The type of ME observed in LC-MS/MS was generally a signal enhancement and an ion suppression for urine and plasma samples, respectively. In the case of SFC-MS/MS, the type of ME randomly varied according to the analyzed matrix, selected column and sample treatment.

## 1. Introduction

Due to its very high specificity and sensitivity, chromatography coupled with mass spectrometry (MS) has become the gold standard for the quantitative analysis of pharmaceutical drugs and their metabolites in biological fluids. In particular, liquid chromatography (LC) hyphenated with tandem mass spectrometry (LC-MS/MS) is nowadays one of the most important analytical platform for bioanalytical [1] and doping control laboratories [2]. The success of LC-MS/MS is based on its ability to accurately and rapidly quantify very small amounts of organic compounds in complex matrices, such as plasma or urine, with a limited sample clean-up prior to injection. The use of electrospray ionization (ESI) has also extended the scope of the technique by enabling the analysis of polar molecules and peptides/proteins.

Nevertheless, the great success of LC-MS/MS also comes with a few drawbacks. In the last years, many researchers have reported that the presence of endogenous compounds, extracted from the matrix and co-eluting with the target analytes, could interfere in the MS source and alter their ionization yield, leading to inaccurate quantitative results. The quantity of ions formed in the source under the influence of matrix entities could either be increased (signal enhancement) or, more frequently, decreased (ion suppression) compared to the case where no

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matrix is present. This phenomenon is known as matrix effects (ME) [3]. In 1993, Kebarle and Tang were the first to experiment and notice the variation in the ESI response when an analyte was ionized in presence of other entities [4]. A short time later, Buhrman et al. published the first assessment of ion suppression when analyzing biological fluids [5]. The exact mechanisms of matrix effects are still unclear, even if several hypotheses have been postulated [5,6]. It has been acknowledged that this phenomenon could be caused by endogenous compounds (phospholipids, proteins, salts…) from the matrix, just as well as exogenous ones introduced during the extraction or the analysis [7].

Even if ME are generally not perceptible during the analysis, since the interference from the matrix is not identifiable on the target analyte selective transition [8], they have to be evaluated during method development. Two different approaches have been proposed to assess ME. First, a qualitative approach consisting in a post-column infusion was developed by Bonfiglio et al. [9]. In this approach, the target analyte is directly infused into the MS detector, while a blank injection of extracted matrix is injected into the chromatographic column. The influence of the matrix is then observed with the variation of the steady response initially obtained by the infused analyte. This approach is convenient because it allows to uncover the segments of signal suppression or enhancement within the whole analysis. Second, a quantitative approach, also called post-extraction addition, was proposed by Matuszewski et al. [10]. In this protocol, the MS response of a target analyte spiked at a given concentration in the previously extracted matrix is compared with the response of the same concentration of analyte in the injection solvent. The difference of signal is then attributed to the matrix effect, and can be quantified by calculating the ratio of both measured values. In the case of plasma, phospholipids have been identified as the major source of ME and can also be monitored during the method development to reveal the areas of possible interference [11].

Several possibilities have been identified to avoid, or at least limit, these ME. The most widespread technique is to use stable isotopically labelled internal standards [12–16]. These molecules differ from the target analytes only by the replacement of some atoms by $^{2}H$ or $^{13}C$ analogs in the structure [17]. As they present a nearly identical chemical structure, they should behave identically to the target analytes during extraction, chromatographic separation and detection, and thus allow correction of the variation of the analyte signal (caused by ME) with an equivalent magnitude of interference of the internal standard. However, these internal standards are often expensive and not always available. Some reports even highlighted that the internal standard and the target analytes might sometimes be affected differently by ME [18]. A second option is to improve the specificity of sample purification, to reduce the presence of endogenous components in the final extract. Several sample preparation techniques exist, providing different levels of selectivity: dilute and shoot (DS), liquid-liquid extraction (LLE), protein precipitation (PP), supported liquid extraction (SLE) and solid phase extraction (SPE) [19–21]. A third alternative is to modify the MS conditions. Indeed, the occurrence of ME can vary according to the source design, the ionization mode and the ionization techniques. Thus, switching from the positive to the negative ESI mode [22], or from ESI to atmospheric pressure chemical ionization (APCI) [14,23,24] can be a successful operation to limit ME. Finally, the chromatographic conditions can be modified to better separate the target analytes from the compounds responsible for ion suppression or enhancement. As an example, the use of 2D-LC [25] or hydrophilic interaction LC (HILIC) [26–30] has been employed to reduce ME. Based on the same idea, the use of supercritical fluid chromatography or subcritical fluid chromatography (SFC), which has been recognized for its orthogonality to reversed phase LC (RPLC) [31], could also be considered as an interesting opportunity to decrease ME. ME in LC-MS have been largely studied and reviewed [3,7,8,14,32–38] in the last decades, but a very restricted number of papers deal with ME in SFC-MS [39–44]. Still, these studies generally highlighted the lower amount of ME in SFC-MS compared to other chromatographic techniques. It should then be interesting to further evaluate this possible advantage of SFC-MS over LC-MS.

The aim of the present paper is to conduct a systematic and comprehensive comparison of ME between RPLC-MS/MS and SFC-MS/MS, when using the positive ESI mode. For this purpose, ME were calculated using the post-extraction addition approach with two different biological matrices, namely human urine and plasma. Each matrix was prepared with a non-selective approach (dilute and shoot (DS) and protein precipitation (PP) for urine and plasma, respectively), and a selective sample treatment (SPE and PP followed by SPE for urine and plasma, respectively). The influence of the SFC column selectivity was also evaluated by injecting all the samples on three complementary columns involving different retention mechanisms (2-picolylamine, hybrid silica and C18). The results in terms of ME were compared between SFC-MS and RPLC-MS.

## 2. Material and methods

### 2.1. Chemicals and reagents

Water of UHPLC-MS grade, methanol (MeOH), isopropanol (IpOH) and acetonitrile (ACN), of OPTIMA LC-MS grade, were purchased from Fisher Scientific (Loughborough, UK). Ammonium hydroxide ($NH_4OH$) and ammonium formate (AmFo) and acetate were purchased from Sigma-Fluka (Buchs, Switzerland), formic acid from Biosolve (Valkenswaald, Netherlands) and perchloric acid 70% from Applichem GmbH (Darmstadt, Germany). Pressurized liquid $CO_2$, 3.0 grade, (99.9%) was purchased from PanGas (Dagmerstellen, Switzerland).

A 10 mM formate buffer was prepared with an adapted volume of formic acid and the pH was adjusted to 3.0 with ammonium hydroxide. The SFC co-solvent was prepared by dissolving 10 mM ammonium formate in a mixture of 98% methanol and 2% water.

### 2.2. Probe compounds

#### 2.2.1. Urine samples

A pilot set of 3 neutral and 37 basic compounds was employed for the determination of ME in urine and consisted of a representative selection of substance of interest in the anti-doping analyses which, in most cases, focus on the analysis of urine samples [2]. The selection covered $pK_a$ values between 6 and 11 with a few rare exceptions, and log *P* values between 0.1 and 2.6, and included the following compounds: amiloride, amphetamine, benzoylecgonine, benzylpiperazine, buprenorphine, bupropion, chlorphentermine, clobenzorex, cocaine, codeine, eplerenone, etilefrine, fencamine, fenetylline, fenproporex, fonturacetam (carphedon), isometheptene, methamphetamine, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxyethamphetamine (MDEA), 3,4-methylenedioxymethamphetamine (MDMA), mefenorex, mephedrone, methadone, methylecgonine, methylephedrine, methylphenidate, metoprolol, nikethamide, norfenfluramine, oxedrine (synephrine), pemoline, pethidine, phendimetrazine, prolintane, propylhexedrine, sibutramine, strychnine, terbutaline and triamterene. All compounds were purchased from Sigma–Aldrich (Steinheim, Germany), Lipomed (Arlesheim, Switzerland) or were provided by the Swiss Laboratory for Doping Analyses (Epalinges, Lausanne). Stock solutions of each individual sample were prepared at a concentration of 1 mg/mL in pure MeOH.

#### 2.2.2. Plasma samples

The pilot set used in the comparative experiments involving plasma was selected from the perspective of toxicological analysis, which often utilizes plasma as the sample of choice. The set was composed of 38 basic compounds covering a range of $pK_a$ values between 6 and 11 with a few rare exceptions in the 2–6 range, and log *P* values from −0.3 to 4.7, and included the following drugs: alprazolam, alprenolol, amphetamine, atenolol, benzoylecgonine,