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Matrix effects: Hurdle for development and validation of bioanalytical LC-MS methods in biological samples analyses

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Liquid chromatography-tandem mass spectrometry is a well-established analytical technique and is anticipated that, with only simple sample preparations, high selectivity and sensitivity of tandem mass spectrometry, coupled to quick separation by Liquid chromatography, allows high-throughput confirmation and detection of trace materials, even in the presence of a complex background. Despite these advantages, quantitative analysis with atmospheric pressure ionization or electrospray interface can be affected by ion (or signal)-suppression or ion-enhancement by interfering substances in the biological sample matrix. This phenomenon is called "matrix effect (ME)." Because MEs can affect critical analytical parameters (limits of detection and quantification, linearity, accuracy, and precision), they should be critically investigated during method development and validation processes. This review gives a detailed description of the expected ME phenomena and their evaluation. It also briefly describes present viewpoint of MEs in the analytical field to promote the knowledge of MEs and their handlings.

INTRODUCTION

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been well established as the most sensitive and selective analytical technique for various pharmaceuticals or compounds in biological specimens. The strength of MS/MS lies in the selected reaction monitoring (SRM) mode, primarily the high specificity, sensitivity, selectivity and throughput it allows. However, unknown compounds in the specimen matrix that elute with the compounds of interest can interfere with the ionization process of the mass spectrometer (Taylor, 2005), inducing ion-suppression or ion-enhancement (Xu et al., 2007; Hennandez et al., 2005; Buhrman et al., 1996). Even with these advantages, therefore, many researchers have reported difficulties in validating analytical methods when analyzing very small quantities of analytes in complex samples such as biological fluids; some of these problems are caused by matrix (Buhrman et al., 1996; Tang et al., 1993). The matrix refers to all components in the samples other than the analytes of interest. Some general matrices typically encountered by biological samples include blood, plasma, urine, bile, feces, and tissue samples (Hall et al., 2012). The ME phenomenon was originally described in the early 1990s and occurred owing to co-eluting matrix components, affecting the detection, precision, or accuracy of the method for the analytes of interest (Tang et al., 1993). Matrix-related ion-suppression or ion-enhancement represents a main hurdle in the validation of quantitative analytical methods with LC linked to atmospheric pressure ionization tandem mass spectrometry (LC-API-MS/MS). A Better knowledge of how the matrix effect (ME) can affect the integrity of bioanalytical methods has re-emphasized the need for satisfactory chromatographic separation of analytes from endogenous biological compounds elements in quantitative bioanalysis by LC-MS/MS (Xu et al., 2007). The intrinsic specificity of LC-MS/MS methods results in chromatograms that do not exhibit any interference, although relatively high levels of matrix components are sometimes present (Hernandez et al., 2005). MEs cause difference in the analyte response when analyzed in a biological matrix compared to that in a standard solution. This phenomenon may be described as ion-suppression or ion-enhancement according to whether the response is attenuated or magnified (Annesley, 2003; Jessome and Volmer, 2006). The intensity of ion-enhancement or ion-suppression of an analyte by a known matrix component can be dependent on the physicochemical characteristics of the analyte. For example, components with high polarity likely appear to be affected to a higher degree than less polar components which may be due in part to their co-elution with other polar components inducing MEs (Hall et al., 2012). These unpredictable and inevitable effects are a regular problem for atmospheric pressure ionization (API) sources (Dams et al., 2003; Heller, 2007). MEs can greatly affect the validity of a method, such as reproducibility, linearity, and accuracy, and lead to erratic quantitation. The degree of ME is very variable and can be influenced by the character of sample and can reduce method reliability even for a series of replicates. Because of the unexpected effect of MEs, a variable response can be observed even among different lots of the same sample or with the same method (Trufelli et al., 2011).

Therefore, evaluation of MEs on the quantitative analysis of pharmaceuticals in biological fluids may present serious issues and is sometimes overlooked as an aspect of analytic method development and validation (Matuszewski et al., 2003). The guidelines on validation for bioanalytical method explicitly require the evaluation of the ME (Bansal and DeStefano, 2007; FDA, 2001; EMA, 2011). The exact mechanisms by which matrix components cause ion-suppression or ion-enhancement are still undefined. The general hypothesis is that co-eluting matrix components affect signal strength by a possible competition for variable charges and for access to the droplet surface in gas-phase (GP) emission, thereby changing the ionization efficiency (Cech and Enke, 2000; King et al., 2000). In fact, necessary steps during method development must be taken before MS analysis to achieve coherent detection and quantification. Most matrix components that might induce ion-enhancement or ion-suppression are eliminated by sample clean-up procedures. Sample clean-up methods and more effective chromatographic separations could diminish the presence of matrix components in the analytical system. However, they are often difficult and time-consuming, and can analyte losses (Trufelli et al., 2011). The preparation of analyte standards in an identical matrix-like sample, and the use of internal standards that have similar or nearly equivalent chemical and chromatographic properties to the analyte, may help minimize MEs. Hence, stable isotope-internal standards are typically the best choice, and in many cases, correct for almost all MEs (Xu et al., 2007). Nonetheless, it is worth noticing that labeled standards are expensive and not available for all components (Trufelli et al., 2011). Caution must be exerted in estimating and removing MEs when developing an assay (Jessome and Volmer, 2006). Sufficient sample clean-up will be required for good performance, especially when complex matrices are involved and sensitive methods are needed. It is now fundamental to estimate the MEs on LC-MS/MS assays as a key first step during method development itself since this has a bearing on the scope of method development and validation procedures (Layek et al., 2008; Srinivas, 2009). Thus to develop a reliable LC-MS/MS method, experiments should be performed to understand and overcome these MEs. This paper provides a brief review of the importance of the evaluation and elimination of MEs in the method validation of LC-MS/MS for the analysis of pharmaceuticals in biological samples.

CONCEPT OF MEs

In chemical analysis, the matrix refers to the components of a sample other than the analyte of interest. The matrix can have a significant influence on the way that analysis is conducted and the quality of the data obtained (Patel, 2011; Pitt, 2009). MEs occur when molecules co-eluting with the analyte of interest change the ionization efficiency (MS response of analyte) of the electrospray interface (ESI) between the LC and MS system (Hall et al., 2012; King et al., 2000; Pitt, 2009). This interface is referred to as the "ion source" and it is here that analyte is charged (Hall et al, 2012). MEs may be present in LC-MS analysis even if MS/MS spectra are used for quantitation of the analytes because this phenomenon occurs before the ions reach the mass spectrometer (Pitt, 2009). MEs have been observed in other detection systems such as in fluorescence and electron capture techniques. MEs causing ion-enhancement or ion-suppression have both been reported, resulting in over- and underestimated results, respectively (Taylor, 2005; Niessen et al, 2006). Anything that interferes with either the charging or the desolvation of the analyte may produce MEs. The exact mechanism of MEs is unknown, but it probably originates from the competition between an analyte and the co-eluting, unknown matrix components for access to the droplet surface and transfer to the gas-phase (Kind et al., 2000). Although experts conclude that the exact mechanism of the interference with analyte release into the gas phase by these non-volatile components is unclear, they postulate that this competition may effectively decrease (ion-suppression) or increase (ion-enhancement) the efficiency of formation of analyte ions of interest for the same concentrations in the interface. Thus, the efficiency of the analyte ions to form and separate is very much dependent on the matrix components entering the ESI source (Taylor, 2005). Because MEs are also compound dependent, the physicochemical properties of a compound have a significant effect on the degree of the ME; more polar interference were found to cause the greater degree of ion-suppression while the least polar caused less ion-suppression under the same MS conditions (Bonfiglio et al, 1999). The importance of MEs on the reliability of LC-ESI-MS/MS has been shown in aspects of accuracy and precision (Buhrman et al, 1996; Matuszewski et al, 1998), and when ion-suppression occurs, the sensitivity and LOQ of a method may be negatively affected (Buhrman et al, 1996). MEs and selectivity issues have been strongly associated with analytical techniques in the biomedical and pharmaceutical fields. Thus, to develop a reliable HPLC-ESI-MS/MS method, experiments should be performed to understand and evaluate these MEs in the LC-MS and LC-MS/MS method development and validation processes (Taylor, 2005; FDA, 2001; EMA, 2011).

MECHANISM OF MEs

The mechanisms of MEs that arise at the interface between the LC and the MS system are not fully understood (King et al., 2000), but they are probably due to effect of co-eluting compounds on the analyte ionization (Trufelli et al., 2011). Thus, ME-free analyses are impossible with the use of LC-MS/MS. The principals of MS detection dictate only charged (positive or negative) gas phase ions are detectable and anything that interferes with either the charging or desolvation of the analyte will produce an ME (Hall et al., 2012). Whereas there are many hypotheses to explain the processes that cause ion-suppression, information on ion-enhancement causes is still lacking (Trufelli et al., 2011). Ion-enhancement describes the increase in ionization yield that occurs with the use of a complex matrix compared

to matrices termed "pure", "neutral", or "inert" based on their constituents. However, it should be noted that LC-grade solvents such as methanol, acetonitrile, and water also interact with analytes during ionization, because of the inherent chemical properties of these compounds and the impurities that exist in them (Annesley, 2007; Napoli, 2009; Keller et al., 2008; Guo et al., 2006). Even though ion-enhancement is less common than ion-suppression, major ion-enhancement of two pesticides, carbendazim and thiabendazole, were found in apple extracts analyzed by LC-ESI-MS/MS (Zrostilkova et al., 2002) and various pesticides in the same extracts of food materials by GC (Poole, 2007). The ME was first described by Kebarle and Tang (1993) and they demonstrated how the analyte signal in ESI-MS can be strongly influenced by the concomitant presence of other electrolytes in the electrospray solution. MEs caused a difference in response for a compound analyzed in a biological matrix compared to a standard solution. Many different mechanisms of ion-suppression have been proposed, but most are specific to the ionization method used (Jessome and Volmer, 2006; Matuszewki et al., 2003). The two main popular API techniques for LC-MS/MS are ESI and atmospheric pressure chemical ionization (APCI). In fact, although MEs can have potentially bad effects on ESI and APCI, the evidence indicates that the ESI interface is more likely to be affected (Matuszewki et al., 2003; King et al., 2000). The ESI process that leads to the formation of gas phase ions involves several steps: (a) addition of a charge to the analyte in the liquid phase, followed by (b) dispersal of a fine spray of charge droplets, followed by (c) solvent evaporation and droplet fission and (d) ion ejection from the highly charged droplets (Figure 1) (Ho et al, 2003; Trufelli et al., 2003; Cole, 1997). Any event that might diminish the production rate of droplets, and eventually the formation of the gaseous phase ions, could contribute to the ion-suppression phenomena. Hence, mechanisms that cause decreased ionization efficiency can be subdivided into two separate processes that occur in the liquid phase and gaseous phase (Trufelli et al, 2003). With the use of a dual ESI-APCI and dual-sprayer ESI source, the major contribution to ion-suppression is from the liquid phase rather than gaseous phase (King et al., 2000). Co-eluting compounds can interfere with the analyte signal in the liquid phase by following four different mechanisms, which are mainly dependent on their physicochemical properties.

The first mechanism responsible for MEs in the liquid phase is based on competition for the available charges and an access to the droplet surface between the analyte and the other matrix compounds (Cech and Enke, 2000; Zhou and Cook, 2001). The presence of impeding compounds at high concentration can increase the viscosity and the surface tension of the droplets, thereby changing the efficiency of their formation and evaporation which, in turn, affects the amounts of charged ions in the gaseous phase that finally reaches the mass analyzer (King et al., 2000; Mallet et al., 2004). To compare the performance of ESI-MS and APCI-MS, non-volatile additives, such as ammonium sulfate, can be used for signal suppression through the formation of solid analyte-inclusion particles (King et al., 2000). The last possible mechanism occurs when matrix components or mobile-phase additives act as ion-pairing reagents with the preformed analyte ions (Zhou and Cook, 2001; Holcapek et al., 2004). The mechanisms responsible for ion-suppression in the gaseous phase are various. The analyte can be transferred into the gaseous phase as an ion or a part of a charged solvent cluster. Once in the gaseous phase, the charge can be lost through

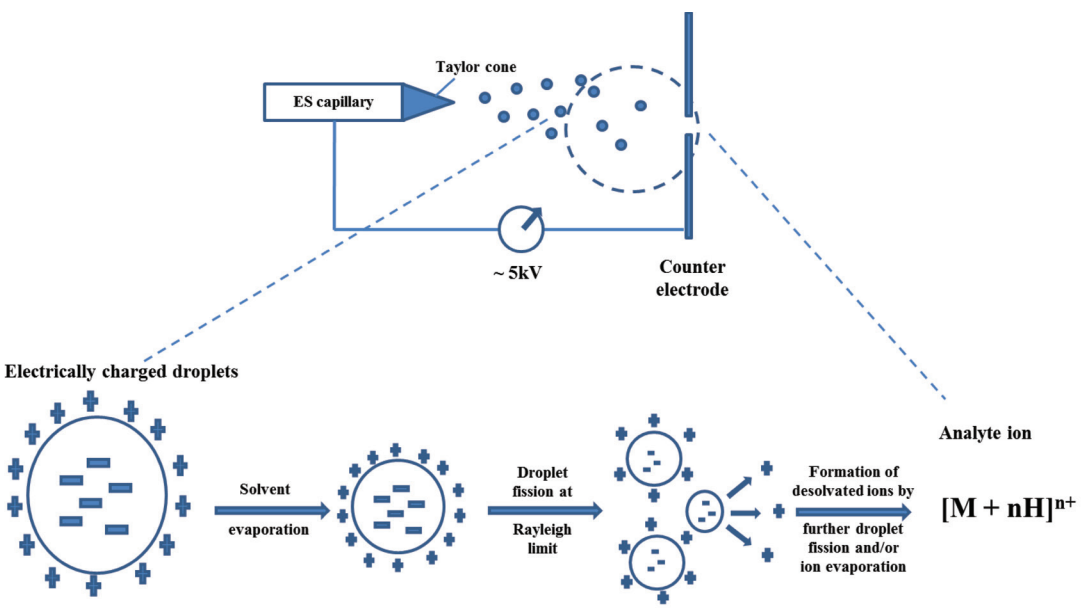


FIGURE 1 | Principles of High-Energy Collision-induced Dissociation of Macromolecules under Electrospray Ionization Process (Cole, 1997).

neutralization reactions, or charge transfer due to the presence of interfering compounds or solvents with high gaseous phase basicity (Tang et al., 1993; King et al., Amad et al., 2000). Understanding the mechanisms responsible for MEs in ESI and APCI represents a fundamental step in the identification of matrix constituents that can be potential source of MEs during the development of an LC-API-MS method.

The sample preparation techniques applied in a method may thus contribute to the ion suppression seen during a run (Annesley, 2003). Typically, laborious methods such as solid-phase extraction (SPE) and liquid-liquid extraction (LLE) achieve short periods of ion-suppression, whereas simple protein precipitation protocols are often associated with more extended periods of ion-suppression due to early eluting, low molecular weight matrix constituents (Annesley, 2003; Vogeser and Seger, 2010). MEs depend on the mode of ionization used as well as on the polarity of the selected ion. Generally, positive-ion ESI is more prone to ion-suppression, whereas negative-ion ESI, APCI, and atmospheric pressure photoionization (APPI) are less affected (Taylor, 2005).

SOURCES OF MEs

Ion-suppression and/or ion-enhancement caused by sample matrices have become one of the fundamental causes of failures and errors in bioanalytical method development and validation. Their sources are extremely diverse and are anlayte-, LC-MS/MS method- and ion source-dependent. Therefore, great care should be taken during method development and validation to identify potential issues (Taylor, 2005; Trufelli et al., 2011; Matuszewski, 2006). Various molecules can lead to MEs, especially if they are present in high concentrations in the extract fraction (Trufelli et al., 2011). The interfering substances may be subdivided into two groups (Antignac et al., 2005). The first group is represented by the so-called "endogenous suppressors," which are intrinsic substances in the matrix and found in the final extract. This group includes salts, highly polar compounds, surfactants, and various organic molecules such as lipids, peptides, and various metabolites with a chemical structure similar to that of the target analyte. The second group is represented by the so-called "exogenous suppressors," which are not originally present in the sample matrix, but may come from various external sources, such as sample preparation and chromatography steps (Table 1).

The compounds causing MEs are often not ionized in the ESI source and therefore cannot be detected by MS. Co-eluting compounds causing MEs can be of different origin. Firstly, ionic compounds in the solvent front near the dead point of the chromatogram may cause ionization suppression if the analyte is eluted too close to this region (Dams et al., 2003; King et al., 2000). Secondly the compounds causing the change in the analytes ionization efficiency may be present as normal chromatographic peaks (Stanke and Alder, 2009). Choi et al (1999) observed 10%-30% ionization suppression caused by the late-eluting components from the previous run. Obvious ion suppression has also been observed after 9 injections of human plasma due to saturation with endogenous compounds, that were initially trapped on the column but elute after several injections (Lagerwerf et al., 2000). Also, the decrease of MS response due to the contamination of the ion source (with gradual decrease of the IS response with increasing sample sequence number) was also observed (Pitt, 2009; Tan et al., 2009). During the bioanalytical method development and validation processes, many of the exogenous MEs are dealt with by careful evaluation of the reagents and supplies used for sample collection, preparation, and analysis. A limited number of extensive and systematic studies have been performed to assess the broad characteristics of MEs in general and method for removal of MEs (Mallet et al., 2004; Chambers et al., 2007; March et al., 2009; Ismaiel et al., 2010). In contrast, most of the current knowledge of MEs is based on studies that focused on specific MEs. For example, phospholipids, and in particular glycerophosphocholines and Lysophosphatidylcholines, represent the major class of endogenous compounds causing significant MEs (Ismaiel et al., 2007; Little et al., 2006). Phospholipids are important classes of biological compounds

TABLE 1 | Various sources of ME

Sources	Explanation
Endogenous components of sample matrix	Carbohydrate, lipids, phospholipids, proteins, bile salts, etc.
Exogenous components	Formulation excipients, leachable components from lab ware, anticoagulants, analyte stabilizers, reagents for sample preparation
Degradation products of analyte	Degradation products of prodrugs and components sensitive to pH, temperature, or light
Impurities and salts	Contained in analyte and internal standard compounds
Poor recovery of analytes	Binding to biological matrices or sample containers
Solvents and additives	Used for LC
Xenobiotics and their metabolites	Analytical samples such as other drug present in patients' blood sample

Modified to references (Hall et al., 2012; Trufelli et al., 2011; Pitt, 2009)

containing one or more phosphate groups. These inherently polar lipids are of great importance for the roles in structure and function of cell membranes, and they are the most abundant of the membrane lipids. Their molecular structures exhibit two major functional group regions: a polar head group substituent, which includes an ionizable organic phosphate moiety as well as other polar groups of various types, and one or two chain fatty acid ester groups, which impart substantial hydrophobicity to the molecule. In particular, the high ionicity of phospholipids is responsible for their impact on analyte ionization in electrospray MS sources (Shen et al., 2005) and on the desolvation of the LC eluent droplets in ESI-MS analysis (King et al., 2000). Therefore, the removal of phospholipids represents a greatly important step in the sample preparation step for LC-ESI based MS (Chambers et al., 2007). In every day practice, ion-suppression is often overcome using empirical methods for such LC-MS/MS analyses, substitution of appropriate HPLC columns, stricter sample preparation, and optimization of lab wares and reagents without identifying or fully exploring the inherent causes of MEs (Hall et al., 2012).

ASSESSMENT OF MEs

There are two common methods for detecting and evaluating the degree of MEs on an HPLC-ESI-MS/MS method: the post-extraction spike method (Matuszewski et al., 2003; Matuszewski, 2006) and the post-column infusion method (Bonfiglio et al., 1999) (Figure 2).

The post-extraction spiked method quantitatively evaluates MEs by comparing the responses of the analyte in neat solution to that of the analyte spiked into a blank matrix sample that has been carried through the sample preparation process (Buhrman et al., 1996; Annesley, 2003; Matuszewski et al., 2003 and 1998; Chambers et al., 2007). The difference in response between the post-extraction sample and the pure solution divided by the pure solution response determines the degree of ME occurring to the analyte in question under the chromatographic conditions. Matuszewski et al. (2003) proposed several protocols to evaluate MEs through the postextraction spike method and they were also the first to introduce the terms ”relative" and “absolute" MEs. The absolute ME was considered as the difference between responses of the same concentration of standards spiked before and after extraction of matrix (Matuszewski et al., 2003; Silvestro et al., 2013). However, even more important is the evaluation of the relative ME, which is the comparison of ME values between different lots of biological fluids and should be investigated in biofluid samples from at least five different sources (Matuszewski et al., 2003). Otherwise, the absolute ME will affect the accuracy of the method, whereas the relative ME will affect the precision and accuracy of the analytical method. The validity of quantitative data obtained with an LC-MS method should be verified by assessing absolute and relative ME (Cappiello et al., 2008). Buhrman et al. (1996) were the first to use the postextraction spike method for the comparison of two different sample pre-treatment methods such as SPE and hexane LLE in the analysis of the platelet-activating factor receptor antagonist (SR27417) in human plasma. The post-extraction spike method provides a more quantitative assessment of an ME. One technique involves extracting two sets of samples; one set contains the analyte spiked in an extracted matrix (post-extraction sample), and the other contains the analyte in mobile phase, solvent, or buffer

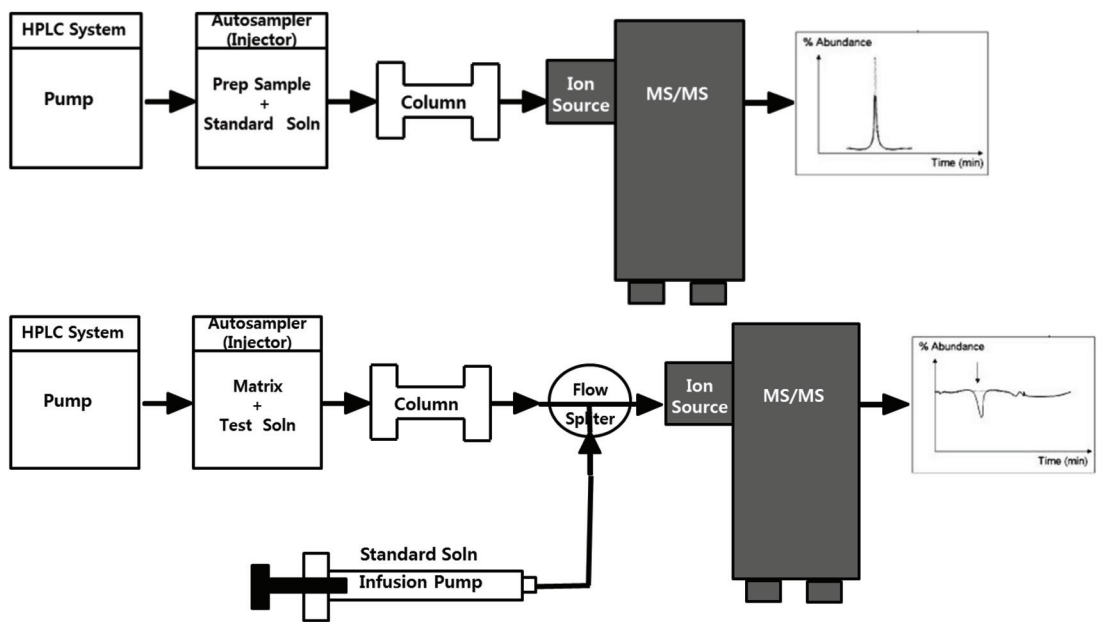


FIGURE 2 | Schematic diagram of two commonly used methods. (A) post-extraction spiked method and (B) post-column infusion method to assess MS in LC-MS/MS.

without going through the extraction process (external standard solution). Both set of samples are prepared with equivalent concentrations of analyte and then processed identically. One can quantitate the degree of ion-suppression or ion-enhancement caused by MEs by use of the following equations (Buhrman et al., 1996; Matuszewski et al., 2003);

Matrix effect (ME. %) = $\frac{A}{B}$ x 100
Recovery of extraction process (RE, %) = $\frac{C}{A}$ x 100
Overall process efficiency (PE, %) = $\frac{C}{A}$ x 100 = $\frac{ME \times RE}{100}$
where, A represents the average peak area of external standard solution (n = 5), B represents the average peak area of a plasma extract spiked at the equivalent concentration of the standard (n = 5), and C corresponds to the peak area for standard spiked before extraction.

The extent of ME is defined as 100-ME(%) (Silvestro et al., 2013). If ME(%) = 100, no ME is present; if ME(%) > 100, there is an ion-enhancement; and if ME(%) < 100, ion-suppression occurs. It is important to point out that the RE (%) represents a "true" recovery that is not affected by the matrix. The presence and entity of the MEs can also be evaluated, when possible, by a direct comparison of the standard line slopes when different HPLC-MS interface (APCI and ESI) are used for the assay of the same compounds, using the same I.S., the same sample preparation and the same chromatographic conditions (Matuszewski et al., 2006). MEs can be also evaluated by the comparison of the slopes of the calibration plots built for standard analytes and for the standard additions performed on the sample (Chen et al., 2013; Gosetti et al., 2010). For example, MEs were studied by comparing solvent calibration curves and matrix-matched calibration curves in the analysis of pesticide residues in ginseng, which contains amino acids, carbohydrate, ginsenoside and volatile oils. This is a challenging situation, due to the high complexity of the matrix, the low concentrations of analytes and the wide range of physicochemical properties involved. In this study, MEs were calculated from the ratio of the slopes of the calibration curves obtained in the matrixes (maize free of pesticides) and in pure standard solutions, which were then multiplied by 100 to get the ion-enhancement or ion-suppression in percentage (Cunha and Fernandes, 2011). The majority of the analytes, about 64%, reported ion-enhancement of between 100% and 150%, while 26% showed ion-suppression. Among 39 pesticides in ginseng, alachlor and triflumuron were chosen for illustrating the positive (ion-enhancement) and negative (ion-suppression) effects, respectively (Figure 3). For determination purposes, therefore, the generalized use of the external standard calibration plot often gives biased results, especially in the analysis of complex samples such as food or biological fluids (Gosetti et al., 2010).

The post-column infusion method provides a qualitative assessment of MEs, identifying chromatographic regions most likely to experience MEs. The assessment is carried out by monitoring the instrument response to a constant infusion of analytes, after injecting an extract into the LC-MS system. A constant flow of a solution that contains the target analyte is delivered into LC eluent at a point after the chromatographic column and before the mass spectrometer (Bonfiglio et al., 1999). Briefly, an infusion pump delivers a constant amount of analyte into the LC stream entering the ion source of the MS. This method is a fast and easy technique that can be used to qualitatively identify regions of ion-suppression or ion-enhancement in a particular ME. In this technique, an extracted matrix sample is injected onto the HPLC column using the LC-MS/MS method for the analyte, while a steady flow of that analyte is infused into the effluent flow between the column and the MS source. Since the analyte is infused into the MS at a constant flow, a steady ion response is obtained as a function of time (Kaul et al., 2010). Additionally, a blank solution such as water, buffer, or the initial mobile phase mixture must also be injected to determine the baseline of the analysis. The regions of ion-suppression or ion-enhancement can be visualized in the resulting chromatograms by comparing the baseline obtained from the blank with each of the matrixes tested (Hall, 2012). Any endogenous compound that elutes from the column and causes a variation in the ESI response of the infused analyte is seen as an ion-suppression or ion-enhancement in the response of that infused analyte (Bonfiglio et al., 1999; Bakhtiar and Majumdar, 2007). If several compounds are determined in one method, all compounds should be infused separately to investigate the possible MEs on every analyte. Moreover, because the ME is not of interest for very low concentration of samples, analytes are infused at concentrations higher than the LLOQ (Van Eeckhaut et al., 2009). Results of post-column infusion experiments enable the researcher to evaluate the influence of different sample extraction methods on MEs, the appropriate analytical column, where MEs occur or are absent during a chromatographic run, the mechanistic aspect of MEs, and the influence of mobile

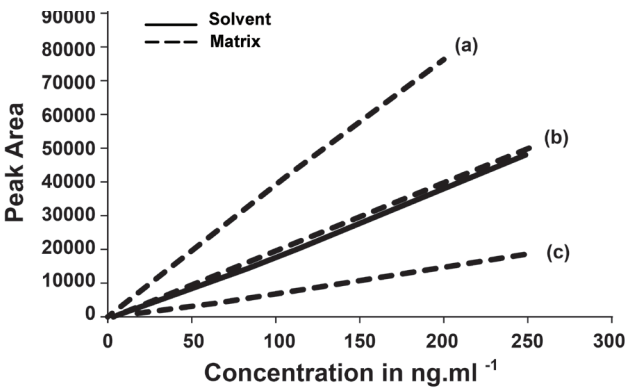
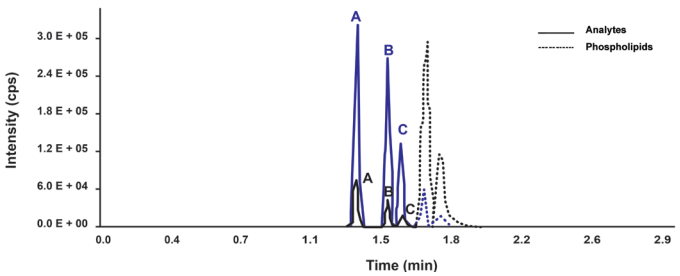


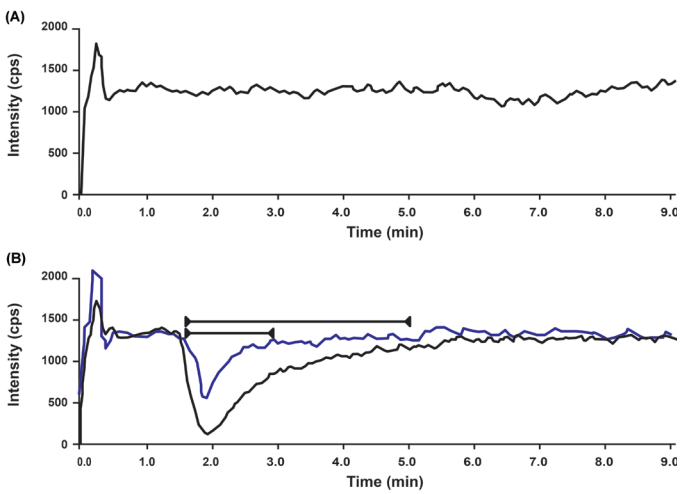
FIGURE 3 | Schematic illustration for comparison of solvent (solid line) and matrix-matched (dotted line) calibration curves, illustrating the presence of matrix effects. (a) positive or ion-enhancement for alachlor, (b) no matrix effect for pirimiphos-ethyl, and (c) negative or ion-suppression for triflumuron (Chen et al., 2013).

additives on response.

As an example, Figure 4-(1) shows mass chromatograms of a spiked human sample after different sample extraction method such as a protein precipitation and Hybrid SPE-precipitation method. The results were very good, ranging from 73.9% to 124.9% confirming the higher efficiency of the Hybrid SPE-precipitation than protein precipitation procedure (Pucci et al., 2009). Figure 4-(2) shows a schematic of a post-column infusion system and a comparison of the injections of mobile phase and a whole blood sample after protein precipitation and SPE preparation in the analysis of sirolimus for therapeutic drug monitoring (Taylor, 2005; Taylor and Johnson, 1998). Sirolimus was monitored by selected reaction monitoring using the mass transition: m/z 931.6 m/z 864.6. The retention time of sirolimus under these chromatographic conditions would be approximately 6 min. For the mobile phase injection (Figure 4-2A), no change in signal was observed throughout the chromatogram. For the sample treated with acetonitrile to precipitate protein (Figure 4-2B-Black), the signal was suppressed for the majority of the chromatogram. The sample prepared by the SPE process (Figure 4-2B-Blue) showed minimal ion suppression in the solvent front but represented constant and maximal response at the retention time of the analyte (Taylor, 2005).



(1) **Post-extraction spiked method**



(2) **Post-column infusion method**

FIGURE 4 | (1) Mass chromatograms of a spiked human plasma sample after (Blue) Protein precipitation procedure and (Black) same human sample spiked with the 3 model analytes at 5 ng/mL after Hybrid SPE-precipitation (Pucci et al., 2009). (2) Comparison of an injection of (A) mobile phase, (B-Black) a whole blood sample prepared by protein precipitation and (B-Blue) by solid-phase extraction in the post-column infusion system. The areas influenced by ME are shown in B and C. The solid lines indicate the region of altered ionization due to ME (Taylor, 2005).

OVERCOMING MEs

The ME may become a hurdle for the validation of a bioanalytical method and, therefore, may detract from the precision and accuracy and lead to a reduction in the quality of the results. Consequently, this is a major issue for the development and validation of bioanalytical LC-MS and LC-MS/MS methods. Hence, removal and/or compensation for MEs are important considerations in the course of method development and validation. Several strategies have been suggested to eliminate MEs, i.e., remove or minimize the presence of co-eluting compounds in the analytical samples. MEs may be reduced by simply injecting smaller volumes or diluting the samples, which is useful as long as instrument sensitivity remains adequate (Heller, 2007). Therefore, the sources of interfering matrix components must also be considered. The interference may come from the current sample being injected, a previously injected sample (as a late eluting interference), or the build-up and overloading of the analytical column by some species (Lagerwerf et al., 2000). Two main methods for removing MEs are optimization of sample preparation to reduce the presence of interfering matrix components in the final extract, and/or modification of chromatographic conditions to shift the retention time to the target analytes away from the area of the chromatogram affected by the MEs (Xu et al., 2007; Hernandez et al., 2005; Niessen et al., 2006). Another method is the use of an internal standard (IS) to compensate for the alteration in signal magnitude. If sensitivity is not an issue, an alternative ionization source, less sensitive to ME, can be used, e.g. APCI (Chambers et al., 2007) or electron ionization (Cappiello et al., 2008). However, it is important to address that there is no universal approach, and in many cases, several technologies must be combined to achieve adequate quantitative results in the development and validation procedures (Trufelli et al., 2011). All together, these more recent topics might speed up the bioanalytical method development and reduce MEs issues at the beginning of the method development (Cote et al., 2009).

Sample Preparation

In general, MEs are directly related to an inadequate sample preparation of the biofluid under investigation. Therefore, a proper sample clean-up method is critical issue in the bioanalytical processes. The choice and

optimization of sample preparation is an appropriate approach to reduce or eliminate the MEs from biological samples before their injection into an LC-MS or LCMS/MS system (Cote et al., 2009). There are a variety of sample preparation methods available for LC-MS, which differ in their efficiency and feasibility to remove interferences for the reduction of MEs. The simplest and fastest method for preparing samples is protein precipitation (PPT). However, it does not result in a very clean extract. PPT is most likely to cause ion suppression in ESI, since this method fails to sufficiently remove various endogenous interferences such as lipids, phospholipids, fatty acids, etc. As a result, co-elution of these compounds with the analyte affects the ESI droplet desolvation process (Heller, 2007; King et al., 2000; Chambers et al., 2007; Bakhtiar and Majumdar, 2007). Such techniques are often specific to the nature of the sample matrix, because different matrices contain different components that need to be removed. The universal sample preparations employed in biological, food, and environmental fields involve SPE and LLE (Trufelli et al., 2011). The extracts obtained from SPE are relatively cleaner than PPT, but the procedures are usually difficult because of operational extent or complexity. Even with an appropriate solvent, a single step rarely extracts the analyte quantitatively, and multiple extraction steps are commonly needed to increase analyte recovery and to obtain cleaner extracts and thus has many pitfalls for LC-ESI-MS (Jessome and Volmer, 2006). Often, a final reconstitution in water at the original pH is needed; with an additional risk of significant loss of analyte. Another issue with LLE is the requirement of a rather large solvent volume (Trufelli et al, 2011). Modified LLE can be used to decrease sample preparation time and improve analyte recovery. In this technique one liquid is immobilized in an inert medium packed into a polyethylene tube and the other immiscible liquid phase is percolated through the immobilized liquid in a manner similar to chromatography. Rapid extraction of the analyte occurs during this intimate contact between the two immiscible phases. The solvent moves through the packing by gravity flow or by use of a gentle vacuum. Compared with classical LLE, novel modified LLEs have resulted in more efficient sample enrichment, shorter sample preparation times, and easier automation (Zwir-Ferenc and Biziuk, 2006). SPE represents the more efficient way to overcome MEs compared to LLE. With regard to the other sample preparation process, SPE has the advantage that many specific protocols can be made to selectively purify the sample from interferences. These strategies are based on two core steps: choice of sorbents that are better suited to the properties of the analyte of interest (Tachon et al., 2008; Souverian et al., 2004) and a suitable selection of the washing and elution solvents (Benijts et al., 2004). Recently, mixed-mode SPE has been made, which is an effective tool for reducing MEs. Mixed-mode SPE uses a dual-retention mechanism, based on reverse-phase coupled with ion exchange, to extract the analyte from the complex matrix in the same SPE cartridge (Chambers et al., 2007). This approach selectively removes matrix compounds from the SPE column, prior to elution of the analyte of interest. In the study evaluating its ability to produce high recoveries of acidic and basic drugs from plasma, the results demonstrated that a clean-up of plasma by mixed-mode SPE lead to minimal MEs for the selected compounds (Mallet et al., 2004). A new sample clean-up technique called Hybrid SPE-PPT has been proposed as a powerful tool to minimize MEs due to the concomitant presence of phospholipids and proteins (Pucci et al., 2009). Recent breakthroughs in sample clean-up have been achieved utilizing on-line sample preparation processes such as on-line SPE and turbulent flow chromatography with valve-switching devices (Trufelli et al., 2011). In addition to LLE and SPE, several other sample preparation tools can be applied in LC-MS, such as ultrafiltration (Petrovic et al., 2002) and microdialysis (Lanckmans et al., 2008). Two alternative approaches could be sample dilution or a reduction of the injected volume (Schuhmacher et al., 2003), however, these approaches clearly appear inappropriate for trace analysis.

Chromatographic Conditions

In order to overcome MEs, improved chromatography is an important way to separate interfering compounds from the analyte. With this approach, the elution conditions are modified to shift the retention time of the analyte far away from the area affected by signal suppression or enhancement. Usually the chromatographic areas that are most affected by interferences are the solvent front, where highly polar and unretained compounds are eluted, and the end of the elution gradient, where the most retained compounds are eluted. Therefore, it is recommended to adjust the chromatographic conditions to elute the target analytes between these two regions (Bonfiglio et al., 1999; Chambers et al., 2007). An simple and effective approach to do this adjustment is to change the mobile phase strength or gradient conditions (Taylors, 2005; Chambers et al., 2007). However, this method generally requires more analytical time, which can be inappropriate in those applications where the development of fast and cost-effective analytical methods is required. Another approach can be the application of a rapid gradient to separate the analyte from the solvent front, while maintaining high throughput (Hopfgartner and Bourgogne, 2003). Therefore, the proper choice of the mobile additives represents a critical step to develop an LC-MS method. The MS/MS response determined as ratio of standard/spiked extracts was used to calculate the MEs in an experiment where formic and acetic acids were added to a group of 35 endocrine-disrupting compounds in water at two concentration level of 0.01% and 0.1% (v/v) (Benijts et al., 2004). The signals of the analytes were significantly affected by MEs irrespective of the concentration of the additives. The addition of weak acidic buffers such as ammonium acetate and ammonium formate to the mobile phase at concentrations of 1 and 5 mM, respectively, resulted in strong ion suppression at the highest concentration (Benijts et al., 2004). Therefore, the ME was compensated for with a proper

sample preparation and the use of stable isotope-labeled internal standards (SILIS). Even if volatile ion-pairing reagents are widely used in LC-UV for improvement of peak shapes and retention times, these can induce severe signal suppression with ESI-MS because of their combined effect of ion-pairing and surface tension modification. A study has emphasized the importance of the composition of the reconstitution solvents to minimize the ME (Zhang et al., 2008). In the assay for the measurement of ziprasidone in brain homogenate/plasma samples, it was found that MEs resulting in ion suppression above 16%-17%, when the composition of the reconstitution solvent was methanol and 20mM ammonium formate (70:30, v/v, pH 3.84 with formic acid). To ameliorate MEs, a change to reconstitution solvent was made (acetonitrile vs methanol vs 20 mM ammonium acetate=48:32:30, v/v, pH 3.84 with formic acid) (Zhang et al., 2008). Hydrophilic-interaction liquid chromatography (HILIC) on silica columns with a low aqueous/high organic mobile phase content is a valuable alternative to reverse-phase LC-MS for the analysis of highly polar compounds (Naidong, 2003; Xu et al., 2007). A sufficient retention can be achieved also to move the more polar analytes away from the solvent front with HILIC columns that provides an increased sensitivity in ESI-MS (Naidong, 2003). However, it is important to note that some polar endogenous components could be strongly retained on a HILIC column, potentially causing MEs that are not observed in reverse-phase conditions (Jian et al., 2010). Recent technological developments in so-called ultra-high-performance liquid chromatography provide significant theoretical benefits in resolution, speed, and sensitivity for analytical applications, particularly when coupled with mass spectrometers capable of high-speed acquisitions (Van De Steene and Lambert, 2008). The improved resolution provides a benefit with respect to MEs through improved separation from endogenous matrices (Marin et al., 2009; Xu et al., 2007). On-line two-dimensional liquid chromatography (2D-LC) provides another efficient way to compensate for signal suppression by its increased resolution and by improving qualitative and quantitative aspects of the analysis of a wide variety of compounds in complex matrices (Dijkman et al., 2001; Rogatsky et al., 2006). High-flow on-line reverse-phase extraction was coupled with normal-phase on silica columns with an aqueous-organic mobile phase LC-ESI-MS/MS to quantify drug candidates in biological fluids (Deng, 2005). Recent studies have proven that miniaturized ESI methods that employ nano-LC columns are more tolerant than conventional ESI toward contaminants in the analyte solution (Van Eeckhaut et al., 2009). It has been shown that reducing the ESI flow rate to the nL/min range leads to increased desolvation, ionization, and ion transfer efficiency over ESI conducted at higher flow rates (Wilm and Mann, 1996). The higher surface-to-volume ratios of the smaller nano-ESI droplets provide not only improved concentration sensitivity but also resistance to ionization suppression phenomena (Trufelli et al., 2011). A high-throughput analysis for SCH211803, an M2 muscarinic receptor antagonist, in human plasma using nano-ESI infusion MS/MS resulted in presented a 4-fold reduction in ion suppression effects, compared to a conventional ESI source operating in the flow injection analysis mode at a flow rate common for LC-MS/MS analysis (Chen et al., 2004). The elimination of large ion suppression effects was achieved by simple chromatographic gradient changes and the use of alternative blood collection tubes and included the use of PEG in the dosing vehicle or in the blood collection tubes (Weaver and Riley, 2006).

Calibration Approach

The standard addition method may be seen as the most suitable approach to compensate for the negative effects of the matrix on analytical method performance, but it is time-consuming and laborious. External sample calibration can be suitable to compensate for MEs from moderately loaded samples with more uniform matrices (Stuber and Reemtsma, 2004). The most widely used calibration method to compensate for MEs uses IS (structural or SILIS). The use of a suitable IS provides one way to balance the variation in the analyte signal with an equivalent disturbance in that of the IS. It was shown that the precision of a method, which used an analog IS can be significantly improved by modifying the mobile phase conditions in such a way that analyte and IS co-elute (Leverence et al., 2007; Kitamura et al., 2001). It appears to be common that SILISs yield better assay performance than other ways of overcoming MEs because they show almost identical behavior to the analyte of interest in sample preparation, chromatography, and ionization processes (Freitas et al., 2004; Ismaiel et al., 2008; Zhao and Metcalfe, 2008). It is of limited use because it is expensive cost and many compounds do not have SILIS analogs that are commercially available. Even if it is generally believed that the application of an SILIS corrects for any ME, this still needs proper attention given during the process of method development and validation (Trufelli et al., 2011; Matuszewski et al., 2003). Even if a SILIS is used, MEs should still be investigated. If ion-suppression significantly lowers the signal of the analyte and/or of the IS, the signal to noise ratio may reduce to a point where accuracy and precision may be negatively impacted (Annesley, 2003). The aim of the authors was to find a more effective method for multi-residue analysis to compensate for MEs even after extensive sample pretreatment and a careful method set-up. The concentrations estimated using the matrix-matched standard calibration with one IS were relevantly similar to those obtained by standard addition, with the concentration ratios close to 100%. These results indicated that the matrix-matched standard calibration with one IS could be a practical alternative to compensate for MEs in multiple component analysis; it is not time-consuming and it is necessary to find an IS for every analyte of interest (Hernandez et al., 2005; Trufelli et al., 2011). An alternative to the IS concept is the echo-peak technique that simulate the use of IS without a SILIS for the analyte of interest. The technique is based on simultaneous injections of reference standards and

samples in one run. The first and second injections are made ahead of, and behind, a pre-column, respectively, thus resulting in a short difference in retention time between the standard and sample peaks (Alder er al., 2004). The echo-peak technique was able to compensate many ME in measurements with both types of ion sources (ESI and APCI ionization).

Mass Spectrometric Conditions

Another strategy to overcome or compensate for MEs is to adjust the mass conditions. MS adjustment can be an advantageous solution because it does not need any change in the rest of the analytical procedure such as sample preparation and chromatographic conditions. In the MS conditions, the occurrence of MEs might differ between ionization modes (positive or negative), ion source types, and ionization technique (Trufelli et al., 2011). ESI and APCI sources are the most commonly used ionization modes. The majority of the validated LC-MS/MS methods reported in the literature were developed using ESI or APCI interfaces (Van Eeckhaut et al., 2009). An application of ionization mode with ESI-MS was a successful strategy to overcome MEs. The negative-ion mode was more specific, and consequently less prone to MEs. Ion source geometries also can influence the extent of MEs (Antignac et al., 2005). Linear geometry instruments were shown to suffer from higher signal suppression than orthogonal or even Z-spray geometry MS (Holcapek et al., 2004). Because APCI is less prone to MEs, a shift from ESI to APCI is an excellent approach to reduce or compensate ME (Matuszewski et al., 1998; Schuhmacher et al., 2003). However, the occurrence of a signal suppression phenomenon has also been dependent on not only ionization mode (APCI or ESI), but also source design (Sciex, Finnigan, Micromass) (Zhao and Metcalfe, 2008; Mei et al., 2003). In the evaluation of both ESI and APCI ionization for the simultaneous analysis of 10 amphetamine-related analytes in meconium, APCI was selected on the basis of few MEs (Kelly et al., 2008). Miniaturized ESI methods are proven to be more tolerant towards contaminations in the analyte solution. Dialysate matrix effects were estimated at different concentration levels of oxcarbazepine and its major metabolite, using a column switching microbore, capillary and nanoLC-MS/MS system (Lanckmans et al., 2008). No clear differences between Ringer's solution and dialysate matrix were observed at medium and high concentration levels of the different methods, but at the lowest concentration level of the microbore system had a significant ME. Atmospheric pressure photoionization (APPI) is a more recent technology and consequently less investigated for MEs but it is less susceptible to ion-suppression and salt-buffer effects than APCI and ESI. The principal benefit of APPI, as compared to other ionization sources, is in efficiently ionizing broad classes of nonpolar compounds (Hanold et al., 2004). LC coupled to direct-electron ionization MS (direct-EI-MS) has been proposed as a valid alternative to the API to overcome MEs for small molecules (Cappiello et al., 2008). This instrumentation allows a straight connection between a nano-HPLC and a MS with EI source forming a solid and reliable union resembling the straightforwardness of a GC/MS system (Cappiello et al., 2007). Different from API, hard-ionization techniques such as EI operate in a high vacuum, high-temperature environment and make use of a physical mechanism that is much less dependent on adverse effects induced by the matrix. Co-eluted compounds are simultaneously vaporized and subsequently ionized by a multitude of independent, single-molecule electron interactions. In the experiment for biological and environmental matrices, pesticides that belong to the classes of triazines and carbamates, and anti-inflammatory drugs, were used as target compounds (Cappiello et al., 2008). MEs were assessed with post-column infusion and post-extraction addition experiments and it provided evidence that direct ESI-MS allowed the analysis of the target molecules regardless of the presence of co-eluted interferences, whereas MEs were always observed with LC-ESI-MS as a combination of ion-enhancement and ion-suppression (Trufelli et al., 2011). Regarding the ionization polarity, the negative mode is usually considered more specific and consequently less subject to ion-suppression, but it is not possible for all analytes (Niessen et al., 2006; Antignac et al., 2005).

CONCLUSION

There is no doubt that MEs can be complicated, time-consuming, and laborious challenges for the analytical specialists charged with developing and validating robust, and reproducible analytical methods. There are various sample and analytical system conditions that cause MEs, and equally, a diverse range of options to overcome them. The fundamental mechanisms that lead to MEs are still not well understood but are still under investigation. In conclusion, it is important to recognize that a careful evaluation of MEs is an essential step in the development and validation processes of analyses based on LC-MS systems. Because of the various matrices encountered, and the unpredictable effects they can have on the final results, it is impossible to propose a universal protocol that could totally overcome or eliminate MEs. This review summarizes our understanding of the idea that by recognizing mechanisms and sources of MEs and, strategies can be developed to assess and overcome MEs. Although MEs seem impossible to eliminate completely, many authors report that a careful sample preparation step with either the application of new stationery phases, or the adoption of more new solutions, is a critical approach in the whole process. When the sample-preparation step cannot minimize MEs, calibration-based approaches are needed to compensate for MEs. The use of an IS, especially a SILIS, is one of the most used ways of doing this; however, there are some limitations such as needing an IS for each analyte in multiple analyte analyses, their expensive price, and the fact that many SILISs are commercially not available. Because researchers agree that an improvement in LC separation is also a good way to reduce MEs, much effort has been addressed to finding the

right mobile phase and column for different applications. It is obvious that the right selection of MS interface can also provide the right solution to overcome the MEs. Therefore, this article has provided a short review of the current perspective of the analytical field on MEs to promote our understanding of them and to help efficiently overcoming them.

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AUTHOR INFORMATION

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