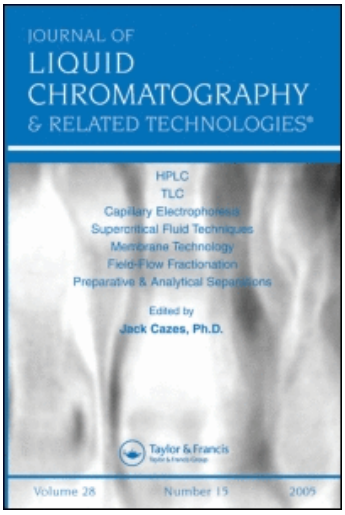

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□ *Despite their enormous utility and diffusion, atmospheric pressure ionization mass spectrometry techniques are subjected to relevant drawbacks called matrix effects (ME). These effects could be summarized in matrix-dependent signal suppression or enhancement that could lead to erroneous quantitative results. The most important method parameters as well as linearity, precision, and accuracy could be modified due to interfering compounds present in the matrix. No validation methods could be accepted without a thorough evaluation of ME and possible strategies to minimize or to correct their influence should be addressed. In this article, we investigate mechanisms that lead to ME and discuss calibration techniques and other strategies to limit these phenomena. Significant examples from different fields of application are discussed as well. Sample preparation procedures, together with instrumental improvements and alternative calibration techniques used by many authors, are reported.*

Keywords LC-MS, matrix effects, quantitative analysis

INTRODUCTION

Liquid chromatography, in combination with atmospheric pressure ionization mass spectrometry (LC-API-MS), represents, without a doubt, the technique of choice in many fields of application spanning from pharmaceutical, biological, environmental, food safety, homeland security, and many others. The availability of a robust and easy to use instrumentation for tandem mass spectrometry (MSMS) has incremented the LC-API-MS performance at trace level analysis, thanks to its high selectivity and sensitivity. Fast chromatography (UHPLC) has further contributed to its success, maximizing high throughput and accuracy. Powerful instrumentation such as electrospray (ESI) and atmospheric pressure chemical ionization (APCI)

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can guarantee satisfactory results even in the presence of complex matrices and interfering compounds in the samples. Sample preparation steps and purification procedures can be simplified, but they are not completely free from important drawbacks. Ion suppression or enhancement induced by interfering compounds coming from the matrix are an obstacle that must be taken into account.[1–4] Matrix effects (ME) represent a severe limitation in quantitative analysis affecting reproducibility, linearity, and accuracy of the methods. They are rather unpredictable because their occurrence is strictly related to the sample nature, and within the same matrix, in different lots of samples. The exact mechanisms are only partially known. Real sample purification prior to mass detection with the aim to obtain a ME free response is a time consuming and complex procedure with many risks of sample losses. A careful selection of the appropriate chromatographic technique could also be helpful to separate analytes and interfering compounds before the detector.

Despite the efforts of many authors, the reasons that cause ion suppression or enhancement are still not fully understood. It is a common agreement that ME are induced by co-eluting compounds, but the real mechanism is only a hypothesis. Although both ion suppression and enhancement were reported by many authors, only ion suppression seems to have a clearer explanation. In 1993 a pioneeristic study by Tang and Kebarle demonstrated the influence of interfering compounds in ESI response, and similar problems were encountered later with APCI.[5] The studies by Matuszewsky et al., Bruins et al., and King et al. demonstrated that ESI is more influenced by ME, due to its ionization mechanism in which the analyte is ionized in the liquid phase before being released in the gas phase.[1,6,7] Signal suppression may occur during all the chain of events that precede the final access of the analytes into the MS analyzer. However, the liquid phase ionization seems more influenced by ME as demonstrated by King and co-workers.[7] Four different mechanisms, strictly dependent on the physicochemical properties of the compounds, could be involved in the modification of the analyte signal:

1. Competition between analytes and interfering compounds for the available charges and for the access to the droplet surface.[8,9]
2. Strong increase of the liquid phase viscosity, in presence of high concentration matrix components, occurring at higher surface tension of the spray droplets that change the efficiency in formation and evaporation of the spray and, as a consequence, of the amount of charged ions in the gas phase that must reach the detector.
3. Ammonium sulfate or other non-volatile additives could be responsible for signal suppression, due to the formation of solid particles.

4. Eshraghi and Chowdhury,[10] Appfel et al.,[11] Gustavsson et al.,[12] Zhou and Cook,[9] Holčapek et al.,[13] demonstrated that mobile phase additives or matrix components could react as ion pairing reagents, leading to the formation of pre-formed analyte ions or neutral complexes.

Various are the ion suppression phenomena that occur in the gas phase, where the analytes could be transferred as an ion or as a part of a charged solvent cluster. In presence of solvents or interfering compounds with a high basicity, the charge can be lost or transferred through neutralization reactions.[5,7,14,15]

APCI is less affected by ME because ionization processes occur in the gas phase.[16–19] Two hypotheses have been proposed:

1. Non-volatile sample components precipitate as solids prior to arrive into the analyzer.
2. The efficiency of charge transfer from the corona discharge needle is strongly modified by the different electron affinity of the analytes and of the matrix components in the gas phase.[18]

It is important to point out that ME are also strictly dependent on the chemical nature of the compounds and that a wide range of molecules can be affected or can be a source of ME. Bonfiglio et al.,[20] studied several drugs and the relationship between their polarity and ME. Their results demonstrated that the most polar compounds were mostly affected by ion suppression. Another work demonstrated the correlation between ME and low molecular weight compounds.[21] Recently Antignac et al.,[2] proposed to divide the interfering substances in two categories: endogenous and exogenous suppressors. The first ones are the compounds present in the matrix and retrieved in the final extracts. The second ones are the compounds not originally present in the matrix, but introduced at some point during method development, such as chromatographic modifiers, phthalates, SPE impurities, labware, etc.

The assessment of ME has been of great interest for method validation, but it is important to evaluate the influence of different matrices also in specific applications. Some authors proposed two strategies to evaluate ME:[20,22–24] post-extraction addiction and post-column infusion. In the first method, two solutions were prepared, one containing the standard of the analyte dissolved in pure solvent and the other containing the analyte dissolved in the sample extract at the same concentration of the standard (the so-called matrix matched standard). If the analyses of the two solutions do not give the same peak area ion suppression or ion enhancement occurred. Buhrman proposed a simple formula to calculate

ion suppression:

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

$$\mathrm{A} = \text{average peak area of the standard solution } (\mathrm{n} = 5)$$

$$\mathrm{B} = \text{average peak area of the matrix matched standard } (\mathrm{n} = 5)$$

This study was the starting point for Matuszewsky and co-workers. They proposed a specific protocol to evaluate ME and introduced for the first time the terms of absolute and relative ME to explain the difference between a standard solution and a spiked post-extracted sample and the difference from different lots of spiked post-extraction samples.[1,23–25] The entire procedure is shown in Figure 1. The authors proposed a modified formula to calculate ME:

$$\mathrm{ME}(\%) = \mathrm{B/A} \times 100$$

Terms A and B are the same of the previous equation. The difference respect to Buhrman's equation is that values <100% mean signal suppression, whereas values >100% mean signal enhancement. Matuszewsky suggested to validate a method when the relative ME, calculated for at least five different lots of samples, has a %RSD <3–4%. In alternative, an internal standard (IS) method must be used, assuming that it shows the same ME profile.

In the post-infusion evaluation method, ME were investigated on the bases of retention time during a column separation of the matrix. A solution of the analyte of interest at constant concentration is infused into the mass spectrometer after the column and during the matrix separation. A schematic view of the entire apparatus is shown in Figure 2. In this experiment, ionization takes place in changing mobile phase conditions

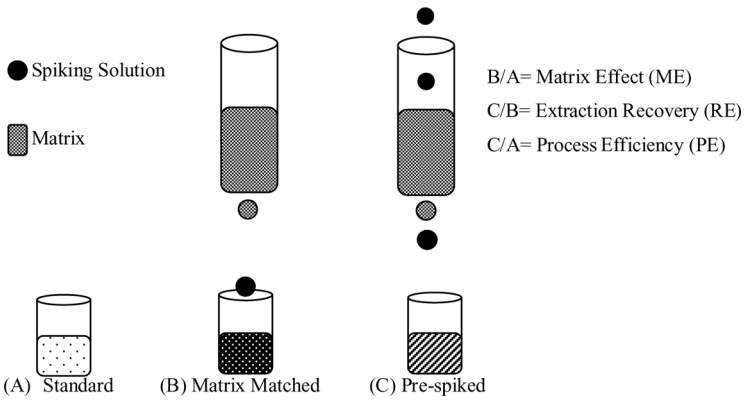

FIGURE 1 Scheme of the post-extraction addition method.

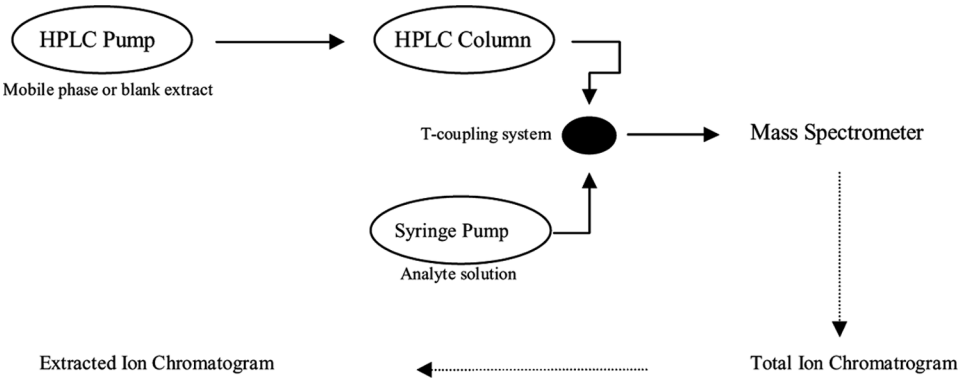

FIGURE 2 Scheme of the post-column infusion system.

as different matrix components are eluted by the chromatographic column. The signal of the analyte is continuously detected by the MS, which reports any variation caused by interfering, co-eluted matrix components. If any of these components induce a drop or an increase of signal, then ion suppression or enhancement can be observed. This approach allows the association of ME to a specific chromatographic method. This could be very helpful, because it is sometimes possible to overcome the problem changing the chromatographic conditions. Although time consuming, this approach is widely used in method development to evaluate the influence of mobile phase modifiers, sample preparation techniques, as reported in the following works: Müller et al.,[26] Dams et al.,[27] Mallet et al.,[28] Souverain et al.,[29] Marìn et al..[30] In post-column infusion, the analytes must be detected one by one, and in the case of a complex mixture, it becomes a very long procedure. On the contrary, post-extraction addition method consents a quantitative evaluation of ME for all the analytes.

As far as the strategies proposed to overcome ME are concerned, many different approaches can be optimized singularly or in combination. There is no universal way able to eliminate ion suppression or enhancement in all the circumstances. The influence of co-eluting matrix components in most cases can be only minimized and data corrections have to be applied. One of the most effective ways to minimize ME is to purify samples prior to the analysis. Many sample preparation techniques can be used, depending on the matrices and on the analytes that need to be detected. Solid Phase Extraction (SPE) and Liquid Liquid Extraction (LLE) are the most commonly used techniques to circumvent ME. Jessome and Volmer[31] used LLE to purify biological samples prior to LC-ESI-MS analysis. The main difficulties that they found were due to pH re-arrangements and solvent volumes needed to maximize the extraction procedure and the signal

response. SPE is more convenient because it requires lower sample volumes and a lower solvent amount. Moreover, it can be easily automated,[32] it could be performed even with on line instrumentations,[33] and, at the present time, it represents the most efficient technique to overcome ME. Sample pre-treatment could be performed with many different sorbent beds or solvents to selectively extract the analytes or elute the impurities, therefore it is easy to find the right combination for most applications.[4,28,34,35] Recently, some authors utilized a combination of SPE techniques to minimize ME: they used reversed-phase coupled to ion-exchange cartridges with good recoveries and very low ion suppression, even with a very complex matrix such as plasma.[26,28,36] Phospholipids and proteins are difficult to eliminate from biological samples. Pucci et al.[37] proposed a new Hybrid SPE-PPT technique to clean up their samples. It consists in protein precipitation (PPT) by acidified acetonitrile, followed by a filtration on a Hybrid SPE-PPT 96-well plate to selectively remove phospholipids.

On line SPE and turbulent flow chromatography (TFC) has been utilized by many authors with good results in minimizing ME, with good recoveries, low extraction costs, and speed of analysis.[33,38,39,40]

Ultrafiltration has been also exploited, as a solution to ME, to eliminate high molecular weight humic substances from groundwater or sediment extracts,[41] but it turned out to be less useful with wastewater samples, because interfering compounds were in the range of low molecular weight ($<$1000 Da).[21]

Schuhmacher et al.[42] adopted the sample dilution and the restricted injection volume as solutions to minimize ion suppression with obvious difficulties in trace analyses.

One of the most satisfactory approaches to overcome ME is to improve chromatography. The aim is to completely separate the analytes of interest from the matrix compounds, to avoid mutual interactions and competitions. There are many ways to change retention times and to separate the components of a complex mixture: different stationary phases, different mobile phases, gradient elution, use of mobile phase modifiers, etc.[3,36,43,44] Changing the chromatographic conditions can also present several drawbacks such as an increase in time of analysis or a worse ionization efficiency.[7,28,45–47] The use of mobile phase modifiers could be responsible of evident ion suppressions as reported by Benijts and co-workers.[34] The use of formic and acetic acid to improve the separation of 35 endocrine disrupting compounds (EDCs) resulted in a signal reduction proportional to the concentration of the additives. Ammonium acetate and formate demonstrated to be deleterious as well. The authors claim that too many ions in the electrospray can reduce the access of the analyte ions to the droplet surface, inducing ion suppression. Many chromatographic separations benefit from ion pairing reagents, but, as in

the previous example, they may induce the same limitations. Gustavsson et al.[12] reported a 30–80% decrease in signal when free formic acid and ammonium formate were added to the mobile phase in the detection of fluorinated carboxylic acids. In the last few years, chromatographic separations could benefit of new ultra high performance instrumentations, called UHPLC. These techniques are fast and very efficient, and they use short columns packed with sub 2 μm particle size to a very high operational pressure. As stated previously, better resolution means better response respect to ME: analytes are completely separated from interfering compounds.[30,36] As well as UHPLC, two-dimensional liquid chromatography (2D-LC) is a valid compromise to overcome ME, keeping analytes separated from matrix components. Despite the good efficiency, 2D-LC is based on high selectivity thanks to the use of more than one column packed with different stationary phases. The works of Pascoe[48] using different chromatographic 2D conditions, and of Deng et al.[49] using high flow on-line reversed-phase extraction coupled with normal phase on silica column, demonstrated the effective reduction of ME, and increased sensitivity for highly polar compounds in LC-ESI-MS.

To limit the influence of complex matrices, the use of appropriate standards to calibrate the system have been suggested. The standard addition method is one of the mostly used techniques, though it is time consuming and not easy to use.[50] External standard method is not as appropriate, and the only calibration approach that seems to have good results in ME minimization is through the use of an internal standard (IS), structural, or stable isotope labeled (SIL). The principle is that the analyte of interest and IS undergo the same procedure and a possible loss of IS can be monitored, calculated, and used to compensate the results. It is extremely important that the physicochemical properties of the IS are similar to those of the analyte.[19,44,51,52] One of the major limitations in the use of ISs, and in particular SIL-ISs, is its cost and availability. Besides this, Liang et al.[16] reported that SIL-IS did not give a good ME correction in ESI and APCI responses for the analysis of some drugs such as methadone, due to a possible interference between standards and analytes ionization. Lindegardh et al.,[53] working on antimalarial piperaquine in plasma, experienced signal suppression for the analyte, and its SIL-IS to a different extent. Numerous other authors found that not all the SIL-ISs (^{17}O, ^{13}C, D, ^{15}N) gave the same results and, consequently, discouraged their use.[54,55] Kang and co-workers proposed different calibration methods in multiresidue analysis.[56] When a large number of compounds have to be quantified, it is quite difficult to find IS or SIL-IS for each compound. The authors used three different approaches: solvent standard calibration with one IS, external matrix matched standard calibration, and matrix matched standard calibration with one IS. The results were compared to those obtained

by the standard addition. Their results demonstrated that matrix matched standards with one IS are the most effective technique to compensate for ME in multiresidue analysis.

Alder and co-workers[57] proposed the echo-peak technique, a new concept to simulate the use of IS. Two injections of the sample and of its standard in a short sequence are performed. In these conditions, standard and analyte are eluted very close to each other and they will be equally affected by ME. The Echo-peak technique gave the same results of matrix matched standard calibration in compensating for ME in pesticides analysis,[58] representing a good alternative to the other calibration methods.

When it is not possible or not convenient to modify the entire analytical procedure, another possibility is to change mass spectrometric conditions. It is well known that ME depend on the ionization technique, source design, or positive/negative ion acquisition. Holčapek et al.[13] investigated ME with five different mass spectrometers, and reported that the linear geometry is much more influenced than orthogonal or Z-spray geometries. Among the various ionization techniques, ESI is more sensitive to ME than APCI, as reported by many authors;[16–19] atmospheric pressure photoionization (APPI) seems to be less influenced by ME than APCI, however, this is a more recent and less-investigated technique.[59]

A completely new instrumentation based on electron ionization (EI) coupled to liquid chromatography, designed by Cappiello and co-workers[60] and called Direct-EI LC-MS has been demonstrated to be free from ME. The concept of this apparatus is well described in a previous work,[61] and it involves a direct connection between a nano-LC system with an electron ionization MS. It is well known that EI is a hard ionization technique that operates in gas phase. All the analytes in the gas phase are ionized by the separate interaction with high-energy electrons emitted by a filament. These interactions are independent from each other and the presence of interfering compounds together with the target analytes do not create suppression in the number of the ions formed. The total ion signals depend only on their concentration inside the ion source. The interface was tested with different sample matrices as human plasma, river water, or seawater, as well as different target analytes.[60–62]

APPLICATIONS

Although LC-API-MS is the most useful and powerful technique to identify and quantify analytes in real samples; it is now clear that in many fields of application, there are significant constraints related to the occurrence of ME. When the analysis of biological or environmental samples come into play (plasma, urine, tissues, wastewater, river water, and other

complex matrices), LC-API-MS often fails due to ME that lead to unpredictable quantification, poor accuracy, and precision.[2,63–68] As stated before, minimizing ME in bioanalytical LC-MS is so important that Food and Drug Administration (FDA) guidelines require ME evaluation in any method validation.[69] Sample preparation and good chromatography could be of great help, as reported in recent works.[31,36,68] Extraction techniques such as PPT, LLE, or SPE are not always useful to eliminate salts, amines, phospholipids, and other endogenous components that could cause ME in plasma and urine.[7,17,31,42,70] Dams et al.,[27] evaluated the synergistic effect of ionization type, biofluid, and sample preparation technique on ME in the determination of illicit drugs. Direct injection, dilution, SPE, and PPT were applied to plasma, urine, and oral fluid samples and analyzed by ESI and APCI. Their results evidenced that ME were different for different biofluids, but always present; inorganic salts were responsible for ME in urine samples; mucin, protein, amino acids, and phospholipids residues induced ME in oral fluids; plasma samples were the most complex matrices, due to the presence of phospholipids and many other components with a wide polarity range. Considering the ionization source, ESI was more affected than APCI. Recent studies demonstrated that phospholipids have the principal role in ion suppression analyzing biological fluids by ESI even with strong extraction procedures.[71,72]

Exogenous interfering compounds can also enter into the method development: plastic vessels (polymers), additives, excipients, or co-present drug formulation in plasma samples can induce ion suppression. To avoid this contamination, it is important to properly select the materials and additives that need to be used.[17,29,44,51,73–76] The most recent overview of validated LC-MS methods for drug analysis in biological fluids is by Van Eeckhaut et al.[68]

Environmental and food analysis is another field in which ME represent a significant drawback. The complexity of the matrices, such as soils, wastewater, vegetable, and food extracts imposes a selective and an efficient sample preparation. In many environmental applications, ME could be minimized by the use of a proper SIL-IS. Two examples of the internal standard approach are reported by Hao et al. and Rodil et al. where several emerging organic pollutants (EOPs) in environmental waters have been investigated.[77,78] Unfortunately, SIL-ISs are not always available or have a compatible retention time.[65,66] As an alternative, standard enrichment can be used: the standard addition method was used to obtain calibration curves for the assessment of ME with APCI[79] and ESI[80] in the determination of biogenic amines in cheese samples. An extensive clean-up of the samples prior to LC-MS analysis represents one of the best possibilities, at the moment, to separate interfering compounds. Selective extraction techniques are often used to reach this goal. Kloepfer et al.[21] used a size

exclusion process to detect benzothiazoles and pharmaceutical in wastewater samples together with nanoelectrospray ion source, less influenced by matrix components.[81] SPE has been widely used to selectively extract analytes from complex matrices. Sometimes SPE gives a valid contribution to ME reduction, as reported in the following articles: determination of carbamazepine in water samples,[82] tetracycline in groundwater,[83] antibiotics in sewage,[84] pesticides in sea and surface water.[85] Van de Steene and co-workers tested numerous stationary phases of different trademarks to find the best solution to ME for the analyses of pharmaceutical in aqueous environmental samples.[86] Humic substances and, in particular, their low molecular weight fraction are very difficult to eliminate, especially in soil samples.[87] Good results have been obtained replacing the common SPE cartridge with molecularly imprinted polymers (MIPs). They are synthetic sorbents with a very high selectivity, utilized in the determination of anti-inflammatory drugs in wastewater samples.[88,89] QuEChERS (quick, easy, cheap, effective, rugged, and safe) sample preparation method also gave good results in the analysis of pesticides in fruits.[90] Different mass spectrometric conditions have demonstrated to be unaffected by ME. Changing ionization technique from LC-ESI-MS to LC-Direct-EI-MS, permitted to detect different classes of pesticides in water samples with no ME.[91,92]

CONCLUSIONS

It is well known that ME represent a complex drawback in LC-API-MS analysis. The mechanisms that rule these phenomena are not completely understood. Any LC-MS operator needs to evaluate ME carefully before developing a method of analysis. ME evaluation has to be included in the method validation, as reported in the guidelines of FDA. Unfortunately, this evaluation is extremely difficult and subjected to the specific application, because different matrices and interfering compounds react differently. In this review article, the possible solutions proposed by some authors to evaluate ME are reported. Based on these studies, it is the general opinion that ME cannot be totally eliminated, but can be reduced at different steps of the method. Sample preparation, involving extraction procedure, sample clean-up, and the use of new stationary phases is the first step to optimize. If this approach falls short, some authors propose to modify the calibration techniques by the use of SIL-IS or internal and external matrix matched calibration standards. Improving chromatography is another way to minimize ME, exploiting a better separation of the analytes from the interfering compounds prior to MS detection. The choice of the instrumentation could influence the results: it has been demonstrated that APCI is less prone than ESI to signal suppression or enhancement.

Electron ionization, as in the Direct-EI interface, gives very good results free of ME in a wide variety of applications.

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