

Pitfalls in LC-MS(-MS) Analysis*

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Introduction

Liquid chromatography combined with mass spectrometric detection (LC-MS) became a powerful qualitative and quantitative technique and is introduced into many toxicological laboratories during the last five years.

Starting from the enhanced selectivity and sensitivity of the MS detector and in view of higher sample throughput misconceptions evolved about speeding up or even elimination of the sample preparation and the chromatographic separation.

However, in numerous reports matrix effects and adduct formation became a major concern. It was demonstrated that these two phenomena can compromise or even invalidate both qualitative and quantitative results. In addition, the build up of searchable spectral libraries also seemed more difficult than expected, because fragmentation can differ from one instrument to another.

In this contribution the focus will be both on matrix effects and adduct formation. After a short description of the mechanism, we will focus especially on how to evaluate and to reduce /eliminate these phenomena. An extensive list of key publications on this subject is also added.

Matrix effect

Mechanisms

Matrix effect can be defined as any change in the ionization process of an analyte due to a co-eluting compound (1). This can result either in an enhancement or in a suppression of the ionization and definitely affects precision, sensitivity and accuracy of an analytical procedure (2).

In electrospray ionization (ESI) the ionization process is taking place in the liquid phase and matrix effects in ESI are due to a competition of matrix constituents and analyte molecules for access to the droplet surface and subsequent gas-phase emission (1). In addition, matrix constituents can also change eluent properties such as boiling point, surface tension and viscosity, all factors known to affect the ionization process (1).

In atmospheric pressure chemical ionization (APCI) the ionization process is taking place in the gas phase and especially the non-volatile matrix constituents are thought to co-precipitate with the analyte of interest, thus influencing the ionization process (3).

Evaluation

For the evaluation of matrix effects basically two procedures are available. One is described by Matuszewski et al. and is based on the injection of three sets of samples (4). Set A consists

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of neat standard solutions (eventually a calibration line), resulting in the reference peak area(s). For set B extracts of 5 different blank matrices are supplemented (after the extraction) with the same amount of standards as used for set A (this then results in peak area(s) B). In addition, set C consists of extracts of 5 different blank matrices, supplemented with the same amount of standards as used for set A (but added before extraction).

The matrix effect (ME%) can then be calculated using the following formula: the area(s) obtained for the set of samples spiked after extraction (B) divided by the area(s) obtained for the neat standards (A) times 100, whereby 100% indicates absence of any matrix effect, whereas < 100% means suppression and values > 100% indicate enhancement of the ionization process.

The recovery (RE%) is then calculated for the area obtained for set C (i.e. spiked before extraction) divided by the area obtained for the samples of set B (spiked after extraction).

Additionally, the process efficiency (PE%) can be calculated using the areas as obtained for sets C and A, respectively. This expresses the total recovery from all sample handling. The three formulas are given below:

$$\text{ME}\% = \text{B}/\text{A} \times 100$$

$$\text{RE}\% = \text{C}/\text{B} \times 100$$

$$\text{PE}\% = \text{C}/\text{A} \times 100$$

Another procedure to evaluate matrix effects is the one described already in 1999 by Bonfiglio et al. (5). This procedure is based on the post-column infusion of a model analyte in a chromatographic run of an extract of a blank matrix. This signal is compared to the signal obtained with the post-column infusion of this same model analyte in a chromatographic run with eluent only, which then serves as the reference signal. This procedure has the advantage to indicate critical areas in a chromatogram. On the other hand, the results are only valid for one single compound and for one level. Typical examples of substances known to influence the ionization process include salts and other endogenous compounds (fatty acids, triglycerides), dosing vehicles (polyethyleneglycol, propylene glycol and cremophore), anticoagulants, and constituents of sampling material (e.g. polymers originating from the sample- and/or extraction tube material, or from the sampling device) (6-8). It is also demonstrated that the ionization efficiency of a co-eluting internal standard is influenced by high levels of the compound of interest.

Applying the Bonfiglio procedure with morphine as a model compound Dams et al. (9) evaluated the combined effect of ionization source type, type of biological fluid (urine, serum and oral fluid), and sample preparation procedure on the matrix effect. Confirming other reports (10, 11) APCI was demonstrated to be less susceptible to matrix effect as compared to ESI. In addition, it was demonstrated that protein precipitation alone resulted in pronounced ME over the whole chromatographic run. SPE was able to remove efficiently hydrophilic interfering compounds but, on the other hand, hydrophobic interferences were concentrated (Fig. 1). When applicable, direct injection or, even better, dilution of the sample yielded less ME (but of course in the latter case, reduced sensitivity).

Strategies to eliminate ME

In the FDA guidelines for method validation (12) the following is advised : “...*In the case of LC-MS-MS based procedures, appropriate steps should be taken to ensure the lack of matrix effect throughout the application of the method*”. However, it is not stated how to evaluate the presence of matrix effect, neither how to eliminate this.

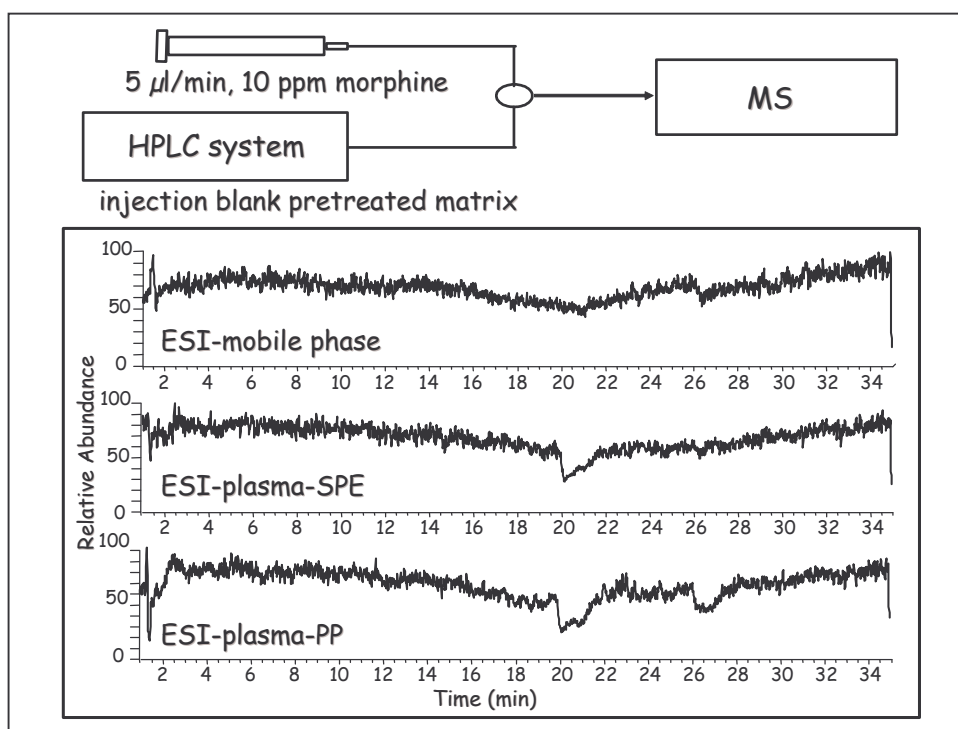


Fig. 1. Post-column infusion model instrumental set-up (top). Compared to the reference signal of the model analyte (in mobile phase) the trace for plasma treated by protein precipitation (PP) shows matrix suppression by hydrophilic and hydrophobic compounds (min 1.5, 20-22, and 26-28). Solid-phase extraction (SPE) can eliminate the matrix suppression by salts but still suffers from suppression by hydrophobic compounds.

First of all, a reduction of the amount of matrix constituents injected can help. This can be done by injecting a smaller sample volume (with subsequent loss of sensitivity) or by applying more selective extraction techniques e.g. based on ion exchange or immunoaffinity chromatography. Secondly, co-elution of the analyte and matrix constituents should be avoided. For this, optimization of the mobile phase can help, but the separation capacity of a liquid chromatographic system is limited as compared to a capillary GC-column (13). Thirdly, the use of matrix matched calibrators is absolutely essential but also this cannot compensate completely for each individual sample (14). The fourth strategy, i.e. the use of co-eluting internal standard(s) seems ideal because it is expected that the matrix effect on the analyte and on the IS is identical. However, as already mentioned, a very high level of the analyte suppresses the ionization of the co-eluting internal standard, and in addition, for multicomponent analysis labelled internal standards are not always available or are cost prohibitive (15). Less common strategies to minimize or compensate for matrix effect include the use of a nano-splitting device (16), echo-peak injection (17), continuous post-column infusion of the internal standard (18) and standard addition to each sample (19). The latter procedure compensates for matrix effect in each individual sample but is very labour intensive for use in routine analysis.

The influence of the mobile phase composition on ionization efficiency is well known in LC-MS. However, mobile phase additives can also have an effect on matrix induced ionization suppression or –enhancement of an analyte (20). In a LC-MS determination of endocrine disruptors in water samples we demonstrated that the addition of small amounts of ammonium formate to the HPLC eluent (1 mM) resulted in substantially better ME% values (less suppression). On the other hand, higher levels of acids (formic acid, acetic acid) suppressed the signal (21).

Adduct formation

Mechanisms

Besides matrix effect adduct formation also adds to the complexity of quantitative LC-MS.

Generally, ESI or APCI result in deprotonated $[M-H]^-$ molecules in the negative mode and protonated $[M+H]^+$ molecules in the positive ionization mode. However, several adduct ions such as $[M+Na]^+$, $[M+K]^+$, or $[M+NH_4]^+$ were also reported in addition to $[M+H]^+$ (22, 23).

Although the exact mechanism involved in adduct formation is not clearly understood, carboxyl or carbonyl ether or ester groups are believed to be responsible for binding the alkali metal ions. Sodium and potassium originate from the biological matrix or from the glass containers used. On the other hand, ammonium ions result from the addition of ammonium acetate or - formate to mobile phases for the LC-MS determination of organic molecules.

Strategies

Difficulties related to adduct formation arise mainly by developing a quantitative procedure because the adduct formation process is not reproducible and consequently it is not clear what adduct ion can be used for multiple reaction monitoring.

A first approach is to measure the adduct ion with the highest response. However, this results in very high (and unacceptable) analytical variations (22). Summation of all adduct ions can help but complicates MS/MS experiments and assumes an equal response factor for all adduct ions.

A second approach involves the attempt to eliminate sodium from the ionization process e.g. by addition of alkali metal complexation products (crown ethers) (24) and by the use of ultra pure deionized water. This is quite laborious due to the ubiquitous presence of sodium, often originating from the glassware, or as an impurity in chemicals and solvents, or due to its presence in the analyzed sample itself. The opposite approach, i.e. the addition of sodium acetate to the mobile phase to enhance the formation of sodium adducts has also been described. However, due to the non volatile character this is not advisable.

Other successful attempts to replace all adducts by one single desired adduct in view of sensitivity and reproducibility have also been reported. In this way reproducible formation of mainly one ion was achieved with the addition of dodecylamine to the eluent (in the case of paclitaxel) (25).

Addition of ammonium acetate results in $[M+NH_4]^+$. The first step in the fragmentation of $[M+NH_4]^+$ is the loss of the neutral NH_3 or of the neutral alkylamine (in the case of alkylammonium adducts) with the formation of $[M+H]^+$. The latter can then fragment further. On the other hand sodium adduct ions are much more stable and yield less fragments. This could point in view of sensitivity, however, the formation of sodium adduct ions is highly affected by the sodium content of a sample (as discussed earlier).

Conclusion

From the above it is clear that the evaluation of both matrix effect as well as adduct formation should be included in the validation procedure of a bioanalytical method based on LC-MS(-MS).

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