Liquid chromatography tandem mass spectrometry in forensic toxicology: what about matrix effects?

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Abstract

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is more and more used in forensic toxicology. This is a highly selective and sensitive technique, but can be negatively affected by matrix effects i.e. the influence of co-eluting compounds on the analyte of interest. The complex composition of forensic samples forces the researcher who is using LC-MS/MS to be aware of matrix effects, evaluate these and, if possible, eliminate these. Therefore, this review discusses the nature of forensic samples and how matrix effects can be evaluated and avoided when analyzing such samples with LC-MS/MS. Moreover, a brief overview of sample preparation and the working mechanisms of LC-MS/MS and matrix effects is given.

1. Introduction

Forensic toxicology uses multiple analytical techniques to determine the presence of xenobiotic components. Screening tests like immunoassays can establish whether there are psychotropic substances present in a sample or not and can eventually provide semi-quantitative values. Confirmatory testing can identify and quantify the specific component. High performance liquid chromatography with ultraviolet detection (HPLC-UV) and especially gas chromatography coupled to mass spectrometry (GC-MS) are widely used for confirmation. LC-MS/MS is being advocated to become the golden standard for both screening and confirmation tests (1). The greatest disadvantage of this technique however is the effect of co-eluting molecules on ionization of the target analyte resulting in ion suppression or ion enhancement, so called matrix effects (1). Therefore, despite the high selectivity of MS, sample preparation and chromatography can not completely be eliminated when using LC-MS/MS. Moreover, studies of matrix effects should be included in LC-MS/MS method validation. Although different strategies to overcome matrix effects have already been published, the purpose of this review is to evaluate the use of LC-MS/MS in a forensic toxicology context by trying to answer following questions: Which type of samples is analysed?; Do such samples need to be treated specifically?; Are there LC-MS/MS prerequisites?; How can matrix effects be evaluated and avoided?

2. Forensic samples

Forensic samples include ante- and post-mortem samples with a heavily variable composition. 1) Different matrices are available. Commonly urine and whole blood are used; alternative matrices are liver, bile, hair, nails, gastric content, vitreous humor, sweat, saliva, brain, bone, fat, muscle or even maggots (2,3). Every matrix has its own characteristics (e.g. pH, typical compounds, …) causing differences between the matrices. An interesting example is the protein content of blood and urine. Whole blood has a high protein fraction responsible for the high viscosity of blood. In urine, the protein fraction is much smaller, ruling out proteins as main interfering compounds. Such differences in composition between matrices apply for other compounds such as sugars and lipids. Moreover, the same matrix from different sources can also greatly vary in composition (4). 2) Both ante- and post-mortem samples may contain a variety of drugs and their metabolites in a wide concentration range. a) Ante-mortem factors that cause variation are the type of drugs used, the administered dose and the victim's metabolism. For chronic users who developed tolerance, toxic doses are known to be higher than for naive users. b) Special attention should be paid to post-mortem samples: the time interval between exposure to a drug and death, post-mortem processes and the time interval between death and sampling can vary causing altered drug concentrations in biological samples. The post-mortem processes include degradation of compounds because of chemical instability or metabolic changes (by both endogenous and bacterial enzymes), redistribution and the appearance of decomposition products (2,3,5). Post-mortem redistribution refers to changed drug concentrations as a result of diffusion of drugs from higher to lower concentrations after cell death. It is an unpredictable phenomenon, but lipophilic and basic drugs tend to be more vulnerable to undergo post-mortem redistribution (2,3,5). Peripheral blood suffers the least from changes in concentrations and should be used instead of heart blood (2,5). Clearly, an unequivocal 'standard matrix' does not exist and forensic samples can be dirty i.e. of complex composition, containing multiple components which possibly disturb analysis. Therefore toxicological analysis should be adapted to the specimens of interest, in order to avoid negative influence of the sample composition on analytical results. In addition special care should be given to the complicated interpretation of concentrations of drugs found in forensic samples, especially in post-mortem samples.

3. Sample preparation

Sample preparation techniques can be used to remove unwanted compounds and/or to concentrate the analyte. Considering the often dirty samples in forensic toxicology, good sample preparation is essential (1,6). Four major techniques are briefly discussed here: sample dilution, protein precipitation, liquid-liquid extraction (LLE) and solid phase extraction (SPE). When using sample dilution (or so called dilute-and-shoot technique), the sample is diluted with an appropriate solvent and directly injected into LC-MS/MS. Protein precipitation is performed by adding an organic solvent, an acid, a metal ion or a salt to the sample which causes disruption of the protein-drug binding and preciptation of unwanted materials (7,8). Protein precipitation with organic solvents such as acetonitrile and methanol is most common in LC-MS/MS, since acids may be detrimental for the analyte's stability and involatile salts are not compatible with LC-MS/MS (7). After mixing and centrifuging, the supernatant is injected into LC-MS/MS. Sample dilution and protein precipitation are two very simple and inexpensive techniques but they result in rather dirty extracts without enrichment (1). For LLE, the pH of a sample is adjusted and analytes are extracted into a suitable organic solvent (1). A back-extraction can further enhance the selectivity. Disadvantages are the use of large volumes of environmentally unfriendly solvents and possible incomplete phase separations resulting in the loss of analytes or dirty extracts (1,6). SPE uses a disposable column containing a stationary phase (e.g. C18, C8, cation exchangers, anion exchangers, ...) that can bind the analytes of interest with a certain selectivity (6). A typical SPE procedure consists of four consecutive steps: column conditioning, sample loading, column washing and elution. SPE is a selective technique, but the used SPE cartridges are expensive and blockage of the of the columns should be avoided (9).

4. LC-MS/MS

Research on the combination of LC and MS started around 1970 and still continues today. Two Nobel prices (Paul, 1989 and Fenn, 2002) and an increasing number of publications involving LC-MS/MS indicate the importance of this analytical technique, not only in forensic toxicology but also in several other scientific fields.

4.1. Chromatography

In LC-MS, reversed phase chromatography is most popular. In this separation mode, the column has a non-polar bonded phase (e.g. C18) and the mobile phase uses polar organic solvents mixed with water (10). Throughout history of LC, particle size of the columns has been decreasing: from 10 µm in the 1970s to the 3 µm range in the 1990s and sub-2 µm nowadays while the instrument's maximum pressure has been increasing (11). The technology using sub-2 µm particles is called ultrafast LC (UFLC), ultra performance LC (UPLC), ultra high performance LC (UHPLC) or rapid resolution LC (RRLC), depending on the manufacturer. The reason for this evolution can be explained using the Van Deemter equation, a formula that expresses the efficiency of separation:

$$H = A + \frac{B}{\mu} + C.\mu$$

Where H is the height equivalent to a theoretical plate, A is the Eddy diffusion, B is the longitudinal diffusion, C is the mass transfer and µ is the linear velocity of the mobile phase (i.e. the flow rate divided by the cross-sectional area of the column). H is a theoretical value to reflect the separation power of a column: a low H value represents an efficient separation. Decreasing particle size lowers the A term since less different paths between the particles can be taken by the analytes and lowers the C term since the diffusion path of the analytes in and out the particles is smaller. The

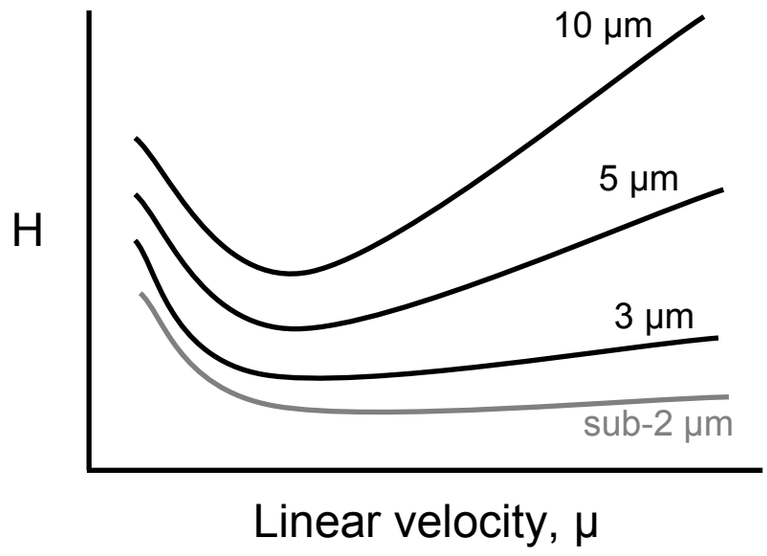

Figure 1: Van Deemter plot comparing columns with different particle sizes. Lower H indicates higher efficiency and sensitivity.

result is a separation with improved efficiency (lower H) and thus improved sensitivity (fig. 1) (11,12). As particle size decreases, the pressure generated in the LC-system increases (up to 1000 bar) (12). Therefore, specialized UFLC hardware that can cope with these high backpressures is necessary because the maximum pressure of a classical HPLC is limited to ~300 bar (12). Since UFLC instrumentation and consumables have a higher cost than conventional HPLC, an interesting alternative might be the use of fused-core technology (12). This technology uses the same principles as UFLC (shorter diffusion path which lowers C-term of the Van Deemter curve and better packing which lowers the A-term of the Van Deemter curve) not by using smaller porous particles as in UFLC, but by reducing the porous part of the particle (12-14). As a result, these sub-3 µm columns can have almost the same efficiency as sub-2 µm columns without the high backpressures, making this technology suitable for classical HPLC systems (12-14).

4.2. Mass spectrometry

Three processes take place in the MS: ionization (in the ionization interface or source), ion separation (in the mass analyser) and ion detection. For ionization, two atmospheric pressure sources are very popular: electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). They operate through a different mechanism making them suited for analysis of different types of molecules (fig. 2) (1).

In figure 3 the working mechanisms of ESI and APCI are schematically illustrated (1,15,16). In ESI, ionization is believed to take place in the liquid phase (15). By applying a strong electrical field, the liquid at the tip of the capillary forms a cone, the so called 'Taylor cone' (15). The solvent evaporates, creating a fine liquid spray of highly charged droplets. The size of the droplets decreases until the repulsive forces between charges exceed the droplet's surface tension, i.e. the 'Rayleigh limit' is reached and the droplet explodes into smaller droplets in a 'Coulombic fission' (15,16). There are two models describing the further process by which gas phase ions originate from the small droplets (16). The charge residue model states that the process of exploding droplets is repeated until a droplet contains only a single analyte. A gas phase ion forms when the solvent from this last droplet evaporates. According to the alternative theory, the ion evaporation model, the electric field on the surface of the small droplets is thought to be high enough to make it energetically favorable for solvated ions to transfer directly into the gas phase. It was argued that the charge residue mechanism dominates for masses higher than 3000 Da and the ion evaporation dominates for lower masses (16). However, the exact mechanism of ESI is still unknown and recent research has suggested that gas phase ionization may also occur (17-19). ESI is known as a sensitive ionization method, however, with a limited linear range. At high concentrations (>~10^{-5} M), a saturation phenomenon is seen and signals no

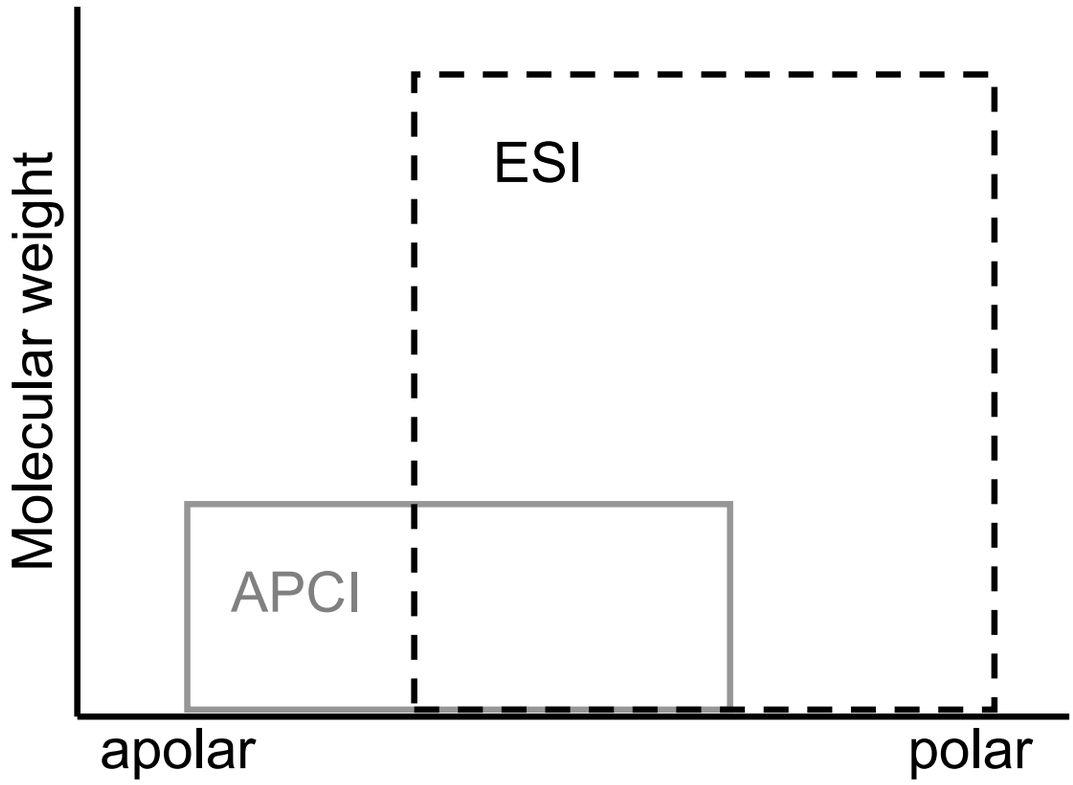

Figure 2: Application area of two ionization interfaces: ESI and APCI. ESI is more suited for polar molecules or compounds with a high molecular weight.

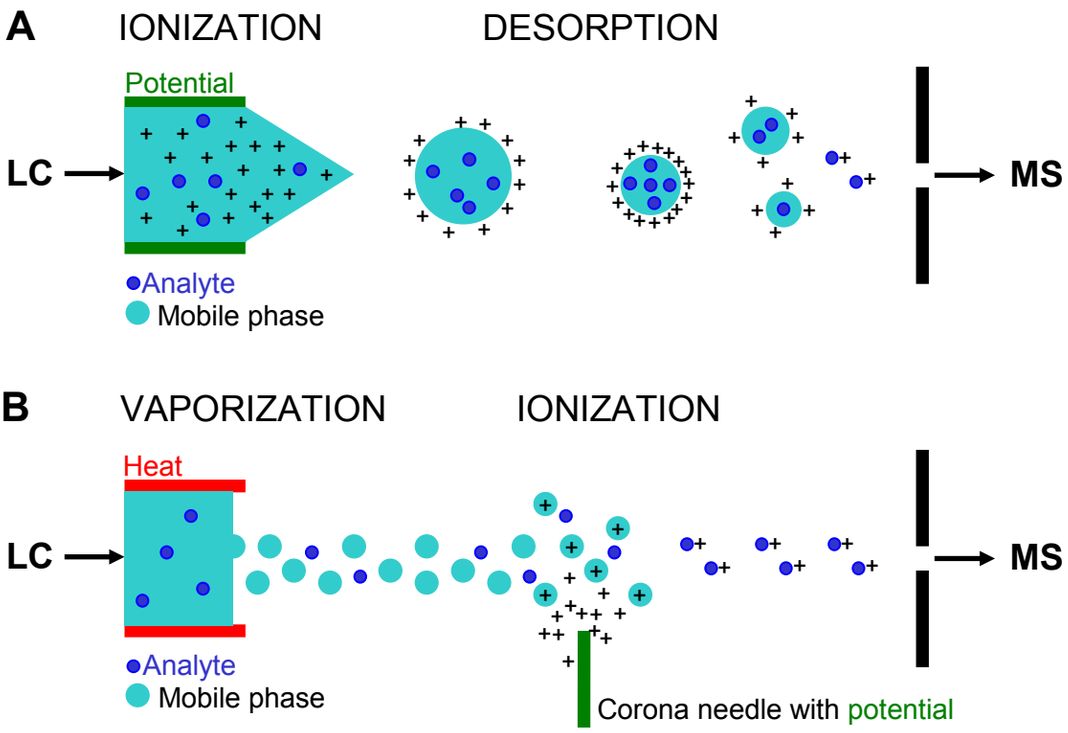

Figure 3: Schematic overview of two ionization interfaces: ESI (A) and APCI (B). (A) In ESI, ionization in the liquid phase (and perhaps also in the gas phase) is followed by desorption (i.e. vaporization because of electrical potential, in ESI possibly assisted by heat and/or gas). (B) In APCI, vaporization by heat is followed by ionization of reagent molecules at the corona needle. The ionized reagent molecules finally can transfer their charge to the target molecules.

longer increase when increasing concentrations (15,20). In APCI, neutral analytes are vaporized by heating of the LC flow (1). The ionization relies on the transfer of charge between a reagent ion formed at the charged corona needle and a target ion (1). The maximum number of ions that can be generated by APCI is much higher than in ESI, because the reagent ions in APCI are formed redundantly. Consequently, the linear range of APCI is wider and, also considering the immediate evaporation of the mobile phase, higher flow rates can be used.

In the mass analyser, the gas phase ions formed in the ionization interface are separated based on their m/z ratios. Common used mass analysers are quadrupole, ion trap and time of flight. LC-MS is a very powerful tool because of the ability to combine two mass analysers (not necessarily the same type) and a collision cell to MS/MS which can be used in different scan modes (1,10,21). Since the type of mass analyser and the used scan mode do not influence matrix effects, a discussion concerning the mass analyzing part is beyond the scope of this review. For more information, the reader can find excellent descriptions elsewhere (1,10,21). The last part of a mass spectrometer is the detector. The most common detection device is an electron multiplier (1).

5. Matrix effects

5.1. Definition

Matrix effects are defined as any change in the ionization process of an analyte due to a co-eluting compound; this can result in ion suppression or ion enhancement (1). It would be better just to use 'signal suppression or enhancement' instead of 'matrix effects', since the co-eluting compounds responsible for this phenomenon can also be other substances than matrix components (22). Matrix effects are mostly observed in the beginning of a chromatographic run, since all polar and non-retained substances elute close to the solvent front (7,22-24). The phenomenon was first described in 1990, but until today the exact mechanism is still unknown (20). Matrix effects are compound-dependent: a study showed that more polar analytes are more sensitive to loss of signal (25,26). And of course, matrix effects depend on the matrix: as the complexity of the samples increases, more and more matrix effects can be seen. Considering the different working mechanism of the two most popular ionization interfaces, the mechanism of matrix effects will also be different for ESI and APCI. Several hypotheses on the mechanism of ion suppression have been formulated and will be briefly discussed here. For ESI, four mechanisms have been proposed: two mechanisms focus on the effect of interfering compounds on the ionization of the analyte

(mechanism 1 and 4), the other two on the droplet formation and evaporation resulting in gas phase ions. Mechanisms 1, 2 and 3 occur in liquid phase, mechanism 4 takes place in gas phase. 1) As described in section 4.2, at high concentrations, ionization saturation occurs. In samples containing interfering compounds, the limit concentration can be easily reached. At that point, the analyte of interest and other substances will have to compete for ionization. Ion suppression is caused by a limited number of charges on the droplet surface or more likely by analytes that are trapped in the centre of the droplet and will not be able to access the surface for gas emission (15). 2) Non-volatile compounds may precipitate with the analyte resulting in solid formation (27). According to the ion evaporation model, precipitates may also prevent the droplets to reach the critical radius and electrical surface field necessary for ion emission and thus limit the transfer of analytes to the gas phase (28). 3) Interferences can change the viscosity and the surface tension of the droplets, thereby reducing solvent evaporation and the ability of the analyte to reach the gas phase (29). 4) Droplets can be contaminated with substances which may evaporate as neutrals. If the gas phase proton affinity of these neutrals is higher than that of the analyte, transfer of a proton from the analyte to the interference may occur in the gas phase (20). APCI suffers less from matrix effects than ESI, since there is no competition between the analytes to enter the gas phase in ionized form (30). 1) Solid formation may be a mechanism of ion suppression in APCI (27). 2) Gas phase reactions such as described in ESI mechanism 4 may cause matrix effects (30). 3) The efficiency of charge transfer from the corona needle to the analyte may be changed by the presence of interferences (30). Although most research has been focusing on ion suppression, ion enhancement may also occur (26,31). Gas phase reactions for both ESI and APCI and changed charge transfer from the corona needle for APCI seem to be possible explanations for this phenomenon. Whether the result is ion suppression or ion enhancement, the co-elution of an analyte with interfering compounds can seriously affect precision, accuracy and sensitivity of a method. Therefore testing for matrix effects should be an integral part of LC-MS method validation, especially in forensic toxicology using often dirty samples. If possible, strategies to decrease matrix effects should be applied.

5.2. Evaluation

To evaluate matrix effects, two approaches are commonly used: a post-extraction addition method and post-column

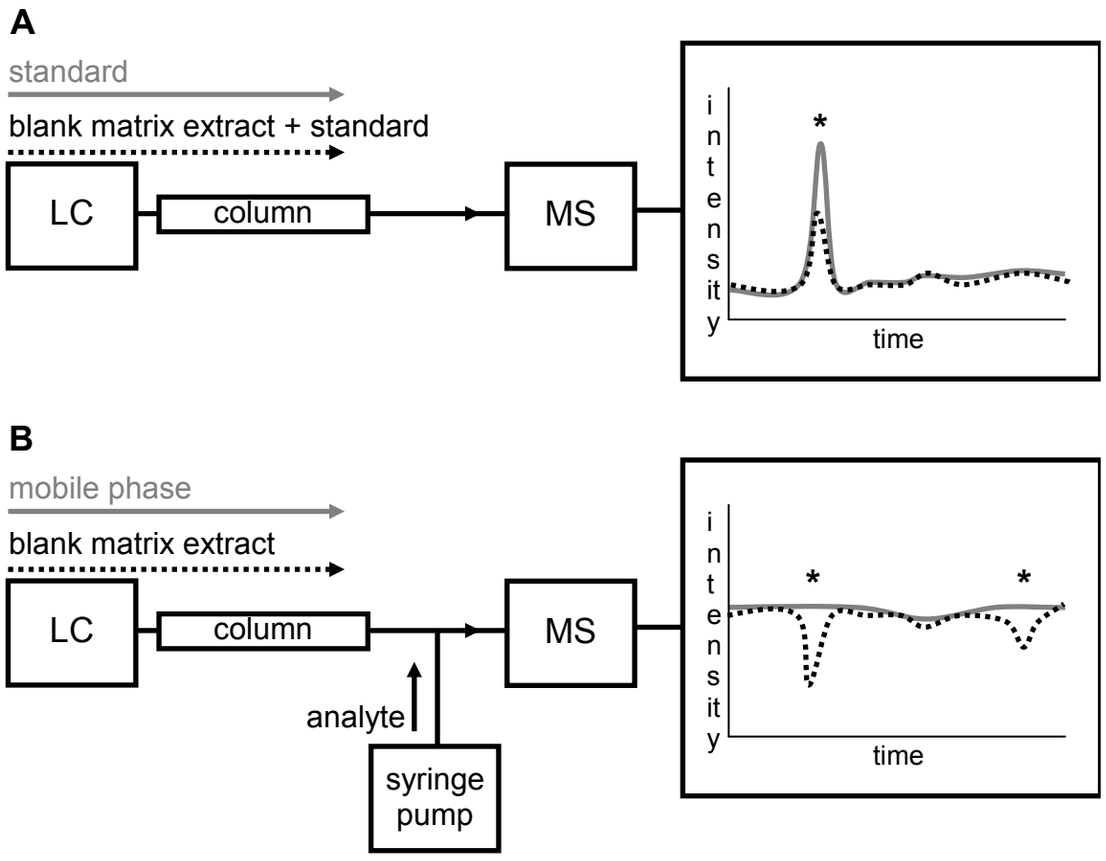

Figure 4: Schematic overview of two strategies to evaluate matrix effects. A region of ion suppression is indicated with an asterix. (A) In the post-extraction addition method, the signals of a standard solution and a post-extraction spiked sample are compared. (B) In the post-column infusion method, there is comparison between a blank sample and a blank matrix extract, which are injected while the analyte is continuously added to the column eluent.

infusion method. In the post-extraction addition method, matrix effects (ME), recovery (RE) and overall process efficiency (PE) can be determined by comparing the response of a standard solution, a pre-extraction spiked sample and a post-extraction spiked sample (fig. 4A) (4). In the post-column infusion system, matrix effects are determined by comparing the signal of a blank sample and a blank matrix extract which are injected into the LC system while the analyte is continuously infused into the column eluent before entering the mass spectrometer (fig. 4B) (25). Post-column infusion gives qualitative information about the matrix effects during the entire chromatographic run, while the post-extraction addition approach gives quantitative information, but only at the time of elution (1). More recently, an approach using comparison of the precision of slopes of calibration curves generated in different sources was described (32). This method is less labour intensive as calibration curves from linearity experiments can be used. However only the variability of the overall process efficiency can be determined and not the values or variability of matrix effects and recovery separately.

5.3. Solutions

Changes in five domains can eliminate, reduce or at least compensate for matrix effects and will be discussed here: the amount of sample, sample preparation, chromatography, mass spectrometry and calibration.

a) Amount of sample

Reducing the injection volume is a simple way to reduce the number of species competing for ionization (7,33). However, sensitivity is reduced. The sample or the extract can be diluted, again the main drawback being the decrease of limits of detection (34).

b) Sample preparation

More extensive sample clean-up and better extraction methods results in fewer co-eluting components and thus less matrix effects. In all papers comparing sample preparation, sample dilution and/or protein precipitation showed the greatest amount of matrix effects, while SPE and LLE resulted in cleaner extracts (23,25,28,35-37). Especially mixed-mode SPE, i.e. SPE that combines two retention mechanisms, was very useful to avoid matrix effects (23,36,37). When using SPE, the protocol should be carefully evaluated, since not only the analytes of interest but also interfering substances can be concentrated, magnifying matrix effects (24). To optimize the SPE procedure, extra wash steps can be added or the elution solvent can be optimised (36-40).

c) Chromatography

Changing chromatography to reduce the degree of co-elution between interfering compounds and the analyte of interest can help in reducing matrix effects. Therefore, the mobile phase can be optimized: changing organic modifier, mobile phase additives, buffers, pH, eluotropic strength or elution profile may reduce matrix effects (7,8,22,33-38). Changing column parameters can also be effective for reducing matrix effects: for example changing column chemistry changes selectivity and using a longer column enhances separation (7). Moreover, when using columns with smaller particles or UFLC, resolution is improved, thus reducing matrix effects (37,40,41). Other researchers used LC/LC, i.e. the coupling of two columns with different retention mechanisms in order to achieve better separation (34). Generally, the higher the flow rate, the higher the level of matrix effects since more organic material requires ionization at the same time (26,42). Flow rate can be drastically reduced by using a nanosplitting device or a post-column split (39,42,43).

d) Mass spectrometry

In general, matrix effects are more pronounced in ESI than in APCI, so changing to APCI might help (4,7,24,27,35). The negative ionization mode is considered more selective than the positive mode since fewer compounds give a signal in the negative mode. Switching to negative mode may lower matrix effects (22). Of course, in this case, the analyte should be detectable in both ionization polarities.

e) Calibration

The appropriate use of a calibration technique will ensure that the calculated concentrations, accuracy and precision of a method are unaffected, but cannot compensate for reduced sensitivity associated with signal suppression. External matrix-matched calibrators (i.e. standards with the same or similar matrix composition as the analysed sample) can be used to compensate for matrix effects (44). However, this requires that the matrix is fairly constant and that a control matrix is available, ruling out its use in forensic toxicology considering the highly variable composition of forensic samples. Internal standards (IS) can be used to compensate for variations in injection, sample preparation, instrumental parameters and also matrix effects (45). An appropriate IS will be affected in the same way by a matrix effect than the analyte, therefore the response ratio of the compounds will remain unchanged (4,7,35,38,39,45,46). Because they possess almost equal physicochemical properties as the analyte, isotopically labelled standards are preferred, but structurally related unlabelled compounds can also be used (45). However, even when using isotopically labelled standards, changes in the response ratio occur (26,31,46-48). This can be caused by the slightest difference in retention time resulting in a different matrix effect on two compounds, different extraction behaviour or mutual influence on ionization of the analyte and the IS (26,31,45-48). Therefore, the IS and its used concentration should be carefully selected (31). The concentration of internal standard should not be too high to avoid influence of the IS on the analyte. However, if the concentration IS

is too low, the analyte can influence the signal of the IS (26,46). Other less often used calibration techniques are echo peak technique, standard addition, extrapolative dilution and (segmented) post-column standard addition. In the echo peak technique an unknown sample and a standard solution are injected in one run, causing the peak of the standard to form an 'echo peak'. Provided that retention times of these two peaks are close enough to be affected by interfering substances in the same manner, matrix effects are compensated (44,49). Since the smallest difference in retention time can cause a difference in matrix effects, this approach may not be optimal for analyzing forensic samples which can contain several interferences. Standard addition is a method to quantify analytes by analyzing the unknown sample before and after addition of standard solution (50). This procedure is time consuming because spiked samples must be run for each unknown. In the extrapolative dilution approach, a sample is repeatedly diluted until the limit of quantitation is reached (51). The concentration of the analyte in each dilution is calculated using a calibration curve based on standard soluitions (51). By plotting the calculated concentration versus the dilution factor, matrix effects can be detected and the correct concentration can be determined (51). This is a labour intensive method since several dilutions for each sample have to be analysed. In forensic toxicology, these time consuming approaches (standard addition and extrapolative dilution approach) might be interesting to study rare cases, analyzing compounds that are not comprised in generally validated methods. In (segmented) post-column standard addition, an internal standard is (periodically) infused into the LC effluent to visualize and compensate for matrix effects using the principle shown in fig. 4B (52,53). Co-elution of internal standard and analyte of interest is not necessary, so isotopically labelled standards are not required and one compound can be used for several analytes, making this approach interesting for multi-analyte procedures (52). However, one should be aware that only the use of an IS can adequately compensate for variable signal loss attributed to both sample preparation and matrix effects. Echo peak technique, standard addition, extrapolative dilution and (segmented) post-column standard addition are best applied to methods where the loss during sample preparation is minimal since they only compensate for altered signals caused by matrix effects. The complexity of forensic samples can cause variability in both sample preparation and matrix effects, making the use of an IS to solve matrix effects more suited.

5. Conclusion

Matrix effects are the major problem of LC-MS/MS. They are dependent on the analyte, the matrix and the ionization interface used. Forensic toxicology is often influenced by these matrix effects, since forensic samples can have a complex compostion, containing multiple components which may possibly disturb analysis. Evaluation of matrix effects should be an integral part of method development in forensic toxicology. If matrix effects occur, adaptations in the amount of sample, sample preparation, chromatography, mass spectrometry or calibration can eliminate, reduce or at least compensate for matrix effects.

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