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Matrix Effects in Mass Spectrometry Combined with Separation Methods — Comparison HPLC, GC and Discussion on Methods to Control these Effects

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1. Introduction

1.1. The evolution of the concept of matrix

Even in the early times of chromatography with conventional detectors (i.e. UV/VIS, FID) it became evident that different sample matrices present peculiar interfering compounds, and the importance of using appropriate spiked matrix calibrators in order to get reliable quantitative results was recognized. In these conditions, however, the main concern was the presence of coeluting compounds giving similar detector responses, while the risk to alter the detector response of the analyte was not yet an issue.

Coupling liquid chromatography with mass spectrometry (LC-MS) was an important step forward because polar and thermally unstable compounds could be effectively analyzed and the poor specificity of previous detectors was overcome. The main steps to the hyphenation of the two separation techniques were made by Doles and Fenn with the development of the atmospheric pressure ionization (API) interfaces (Doles et al, 1968; Whitehouse et al, 1985; Fenn et al, 1989; Mallet et al, 2004). In short time LC-MS/MS has become an important tool for the analysis of drugs and metabolites from biological fluids, or for trace analysis from complex mixtures with many applications, e.g. pharmacokinetic studies of pharmaceuticals or the study of proteomics. John Fenn received in 2002 the Nobel Prize in Chemistry for his contribution to the development of the electrospray ionization (ESI) technique.

This huge improvement in selectivity brought quickly to a simplification of separation methods and/or sample preparation but on the other hand unexpected quantitative or even

qualitative results were observed. Significant differences in peak intensities were observed comparing chromatograms recorded on neat solutions and biological extracts with equivalent concentrations. In most of the cases the signal intensity is reduced, although sometimes signal enhancement could also be detected. A new concept, that of matrix effect, was emerging, and coeluting components were recognized as very important in influencing analytes ionization and detector response. A much more complex vision of the matrix effect is now widely accepted and even matrix differences between samples of the same kind are in the center of attention.

As a matter of fact, a lot of emphasis is currently put on adequate validation procedures for analytical methods in order to be sure that correct quantitative or even qualitative data are obtained.

Matrix components of a sample can affect, most times negatively, the analytical measurement of the main compound. The phenomenon was called "matrix effect" and was defined at the Workshop on "Bioanalytical Method Validation-A Revisit with a Decade of Progress" (Workshop held in Arlington VA, January 12-14, 2000) as "The direct or indirect alteration or interference in response due to the presence of unintended analytes (for analysis) or other interfering substances in the sample" (Shah et al, 2000).

Mass spectrometry is a powerful analytical technique based on ions separation; therefore ionization is of key importance for high sensitivity and selectivity. The ionization efficiency depends on the physico-chemical properties of a molecule, and also on the conditions established in the ionization interface. In ESI the eluent from the chromatographic column, already containing ionic species, is pumped through a capillary; a high voltage is applied to the capillary producing charge separation at the surface of the liquid. The so-called "Taylor cone" is produced at the end of the capillary and liquid is nebulized into charged droplets. When the charge becomes sufficient to overcome the surface tension that holds the droplet together, gas-phase ions are released (Kearle and Tang; 1993, Chech and Enke, 2001). Iribarne and Thomson published one of the first theories on gas-phase ions emission from charged droplets. The rate of ion emission from a droplet is proportional to the number of charges and will be higher for the more surface-active ion (Iribarne and Thompson, 1976 and 1979). It is very likely that here is where matrix components are interfering, competing in these processes; the mechanisms are not fully elucidated.

The ion suppression effect in ESI was first described by Kearle and coworkers in the 1990s. They have shown that the ESI response is linear with the analyte concentration in the range from 10^{-8}M to 10^{-5}M , and in a mixture of organic basic compounds, the signal of an organic base ion measured as MH^+ could decrease with increasing concentration of another basic compound depending upon surface activity and Iribarne constants of the respective compounds. The decrease in ion intensities of the MH^+ ions were attributed to gas-phase proton transfer reactions between the electrosprayed gas-phase ions and evaporated molecules of the stronger gas-phase base (Ikonomou et al 1990; Kearle and Tang, 1993).

Buhrman and coworkers published in 1996 a study on ion suppression in plasma samples (Buhrman et al, 1996). The authors have validated a method for the quantitation of SR 27417 (a

platelet-activating factor receptor antagonist) in human plasma. During method development, noticing a loss of signal in extracted samples compared to the neat solutions they studied the extraction efficiency of three sample clean-up procedures and their effect on analyte ionization. The matrix effect was evaluated by injecting: A) a neat solution of a concentration present in the sample considering an extraction efficiency of 100%; B) a spiked plasma sample extracted and C) a blank plasma extracted and spiked post-extraction with the solution from experiment A). Subsequently, the loss of intensity between A) and B) represents the efficiency of the total process, whilst the loss of intensity between A) and C) is the ion suppression (Buhrman et al, 1996).

Later, Matuszewski and coworkers compared the HPLC-MS/MS interface with a “chemical reactor” in which primary ions react with analyte molecules in a complex series of charge-transfer and ion-transfer reactions, depending of the ionization energies and proton activities of the present molecules (Matuszewski et al, 2003).

In such conditions, as the solvent evaporates, inside the droplet a competition starts between the proton affinity of the analyte and co-analyte, for the proton transfer to take place. If the co-analyte has a higher gas-phase proton affinity than the analyte this one will be protonated first, instead of the analyte, therefore the ion intensity of the last one will be reduced. In the same time, the presence of any nonvolatile matrix components will prevent the droplets to reach their critical radius and surface field by increasing their viscosity and surface tension and decreasing the solvent evaporating rate (Matuszewski et al, 2003). As observed also by King et al, the ionization suppression in biological extracts was the result of the high concentration of nonvolatile compounds present in the droplet solution and was not affected by the reactions occurring in the gas-phase (King et al, 2000).

Matrix effects are not attributed only to ESI interface, although some studies show that atmospheric pressure chemical ionization interfaces (APCI) are less susceptible to ion suppression, mainly due to the APCI mechanism, which occurs by charge transfer from the ionized solvent/additives when the analytes are already in gas-phase (King et al, 2000; Henion et al, 1998; Hsieh et al, 2001, Souverain et al, 2004). Nevertheless APCI and other types of ionization (e.g. atmospheric pressure photoionization – APPI) are not matrix effects-free but the ionization processes being different, the behavior is of course different from that of ESI. Ion suppression is not always directly related to the saturation of the charge available in ESI, but it may be related to changes either in the liquid-to-gas transfer efficiency or in the charge transfer efficiency (Sangster et al, 2004); experimental data obtained also by our group with these three ionization interface will be presented in the next sections.

Using the same sample preparation and chromatographic conditions, some studies compared the results obtained with a triple quadrupole MS interfaced with APCI or ESI, in order to evaluate the selectivity and reproducibility of an existing HPLC-MS/MS assay method (Fu et al, 1998, Matuszewski, et al, 2003).

Matuszewski and coworkers have introduced the concepts of quantitative assessment of the “absolute” and “relative” matrix effect. The absolute matrix effect was considered as the difference between response of the same concentration of standards spiked before and after

extraction of the matrix. The variation of the absolute matrix effect between several lots of the same endogenous matrix was defined as relative matrix effect. Matrix effect (ME), recovery of the extraction (RE) and process efficiency (PE) were evaluated according with the equations:

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

$$RE(\%) = C/B \times 100$$

$$PE(\%) = C/A \times 100 = (ME \times RE) / 100$$

Where A is the chromatographic peak area of the standard in neat solution, B is the peak area of the standard spiked into plasma after extraction and C is the peak area of standards spiked before extraction.

It is the same approach used by Buhrman group, but it also takes in consideration the potential for ion enhancement. In this study, Matuszewski and coworkers observed significant ionization enhancement with APCI interface ($\approx 130\%$) and slight enhancement (analyte) or suppression (internal standards) with ESI interface ($\approx 110\%$ and $\approx 90\%$, respectively) (Matuszewski et al, 2003).

To conclude, the effect on the analytical signal of all compounds excepting the main analyte is therefore defined as “matrix effect” and is expressed as a matrix factor by the equation:

Matrix Factor (MF) = Peak response in presence of matrix ions / Peak response in the neat solution

MF=1 indicates no matrix effect

MF<1 indicates ion suppression

MF>1 indicates ion enhancement.

In bioanalysis, matrix effects are very specific and complex at the same time, because each biological matrix is unique and can affect differently any analytical technique used for the identification and quantitation of an analyte from the matrix. The extent of the matrix effect depends upon: 1) the sample matrix; 2) sample preparation procedure used for clean-up, 3) chromatographic separation (column, mobile phase,...) and, 4) ionization interface.

Phospholipids are a major source of matrix effects in bioanalysis. Most of them are ionized under positive mode due to the presence of quaternary nitrogen atoms. Glycerophosphocholines are the major phospholipids in plasma and are known to cause significant LC-MS/MS matrix ionization effects in the positive mode (Little et al, 2006, Bennet et al, 2006, Jemal et al, 2010).

The quantitative evaluation of the matrix effect is performed based on the approach described above (Buhrman et al, 1996, Matuszewski et al, 2003). For a qualitative evaluation, a classical experiment consists of injecting the extracts of blank (non-spiked) biological samples on the column, in the analysis conditions, while the target analyte is infused post-column at a concentration giving a high and flat signal. The influence of the co-extracted compounds will produce gaps (ion suppression) or peaks (ion enhancement) on the analyte signal. A lot of examples were presented in literature (Bonfiglio et al, 1999; Dams et al, 2003; Souverain et al, 2004 etc); in the second section of this chapter experimental data obtained for pramipexole in different analytical conditions will be presented.

The matrix effect became a critical parameter in bioanalytical method development and validation. For pharmacokinetic studies, FDA guidance documents (FDA 2001) require that this effect to be evaluated as a part of development and validation of a quantitative LC-MS/MS method, and more recent EMA guidelines as well (EMA 2011).

For an accurate quantitation of the requested analytes, the use of an isotope-labeled internal standard is required. This will reasonably compensate the eventual matrix effects, being chemically identical and hence it will be suppressed or enhanced in the same manner as the analyte (Viswanathan et al, 2007). When isotope-labeled standards are not easily available, structural analogues of the compound of interest or related molecules that match its extraction and chromatographic properties can be used, but in this case the matrix effects compensation can be different and the impact on results reliability should be evaluated.

2. Relevance of matrix effect in HPLC-MS/MS

Due to its high selectivity and sensitivity, mass spectrometry in tandem with liquid chromatography became quickly a powerful analytical tool and even took the supremacy over the coupling with gas-chromatography in various fields, like genomics and proteomics, metabolite identification and metabolomics, or regulated bioanalysis. Along with the development of HPLC-MS/MS instrumentation and its applications, the matrix interferences were observed and studied from the beginning. The importance of matrix effects was recognized especially in quantitative analysis, because they can heavily influence the reproducibility, linearity and accuracy of the method, leading to altered results (Trufelli et al, 2011). Although not so much considered, qualitative analysis can be also affected because some trace compounds will not be identified in a sample if their signal is excessively suppressed by matrix, thus giving erroneous assessment of the composition of the sample.

Matrix effects are different depending on the sample nature, and moreover variations are observed between different lots of the same type of sample. The phenomenon was defined by Matuszewski et al as “relative matrix effect”. As discussed above, electrospray ionization is more influenced than other ionization techniques. Coming to chromatography, the matrix effect is usually higher on the early-eluting peaks, because all hydrophilic compounds from the biological sample are not well retained in reversed-phase columns and usually elute in the first minutes. This is not a 100% rule though, because some phospholipids, flavonoids or other classes of organic compounds can be strongly retained and in some cases, depending on the chromatographic conditions, they even accumulate in the column and elute periodically after a series of injections, thus a strong matrix effect being noticed only on the respective sample and not overall (Little et al, 2006; Jemal et al, 2010).

Some examples registered during routine work in our laboratory will be presented next. In the first case, we have developed and validated a method for quantitative determination of salicylic and acetylsalicylic acids in plasma by LC-MS/MS on an API4000 QTrap (AB Sciex) quadrupole-linear ion trap instrument, using an ESI interface, in negative mode. The chromatographic separation involved an Ascentis Express RP-Amide (10cmx2.1mm, 2.7µm) column,

eluted in isocratic conditions, at 0.25 mL/min, with a mobile phase consisting of 0.1% formic acid in water/acetonitrile 45/55 (v/v). The concentration range to be measured in the biological samples being quite high (low limits of quantification/LLOQs of 5 and 50 ng/mL for acetylsalicylic and salicylic acid, respectively), a simple protein precipitation with acetonitrile was chosen for sample clean-up and further optimized. D4-salicylic and D4-acetylsalicylic acids were used as internal standards and matrix effects were evaluated during method validation.

Analysing a large set of plasma samples from a group of patients treated with acetylsalicylic acid, different matrix effects were observed in some volunteers compared to those registered on calibration curves and control samples (prepared by spiking a pooled plasma lot). Fig. 1 and 2 show the metric plots of D4-acetylsalicylic acid and D4-salicylic acid chromatographic peak areas, respectively, in one of the batches (including calibrator and OC samples); as expected stronger ion suppression can be seen on the transition of D4-acetylsalicylic acid, eluting first (retention time 1.15 min), compared to D4-salicylic acid (retention time 1.6 min).

Figure 1. D4-acetylsalicylic acid (internal standard) chromatographic peak area plotted against the sample index in the results table, after the analysis of a batch containing unknown samples, calibration (CC) and control (QC) points. ESI ionization. Data legend on the left. High ion suppression can be observed between different plasma sources (unknown samples versus CC and QC samples).

Another situation often encountered in quantitative determinations is when the analyte signal is progressively suppressed after the injection of biological extracts, until a plateau is reached. For this reason, column equilibration by injecting an appropriate number of extracted samples is recommended before starting the analytical run.

Figure 3 shows the influence of the accumulated matrix on the signal of medroxyprogesterone-17-acetate observed in our laboratory during method development. In this case the analysis was performed on an API 5000 triple quadrupole mass spectrometer (AB Sciex), in positive ions mode, using an APCI interface. Medroxyprogesterone-17-acetate was used as internal standard for the quantitative analysis of chlormadinone acetate. The sample extracts

Figure 2. D4-salicylic acid (internal standard) chromatographic peak area plotted against the sample index in the results table, after the analysis of a batch (the same as in Fig. 1) containing unknown samples, calibration (CC) and control (QC) points. ESI ionization. Data legend on the left. High ion suppression can be observed between different plasma sources (unknown samples versus CC and QC samples). However, later-eluting D4-salicylic acid was less affected than D4-acetylsalicylic acid by matrix effects.

Figure 3. Medroxyprogesterone 17-acetate (internal standard) chromatographic peak area plotted against the sample index in the results table, after the analysis of a batch containing unknown samples, calibration (CC) and control (QC) points. APCI ionization. Data legend on the left. Progressive ion suppression is noticed after the injection of plasma extracts, until an equilibration of the system with the matrix components. The injection of a solvent sample is partially alleviating the matrix effects.

were separated on a LiChrospherRP-Select B (12.5 cmx3 mm, 5 μ m) column, eluted at 1.2 mL/min with a mobile phase composed of acetonitrile and water, in gradient conditions (starting from 70 to 97% acetonitrile). The low limit of quantification being in the low pg/mL range, a liquid-liquid extraction procedure was selected for sample clean-up. As it can be seen on the internal standard peak area metric plot, the sensitivity is quite high in the first samples of the run, then the signal goes down until stabilizing at a certain level. After the injection of a wash sample (mobile phase) the sensitivity increases again. The decreasing intensities could be produced by an instrument charging also caused by matrix components accumulated on some parts of the ion-path. This is an example of ion suppression in APCI and underlines the fact that co-extracted matrix can have an impact not only on the current but also on the next injections.

More recently we have conducted in our laboratory a series of experiments on pramipexole, a dopamine agonist in the non-ergoline class prescribed for the treatment of Parkinson's disease and restless leg syndrome. Because of its structure and its quite low molecular mass (211.324 g/mol), pramipexole quantification has proven to be a difficult problem to solve. Very good sensitivity and chromatographic separation were achieved with neat standards, but going further to plasma samples, issues of ion suppression and high chromatographic background have led to a long method development that covered almost all possible tests. For the final method, a separation on a pentafluorophenylpropyl stationary phase (Discovery HSF5, 10cmx2.1 mm, 5 μ m, Supelco) was preferred, and elution was performed with a mixture of acetonitrile/ammonium formate 10mM, pH 6 (75/25, v/v) delivered at 0.7 mL/min. Mass spectrometer, API 3000 (AB Sciex) with HSID modified interface (Ionics) was operated in ESI positive ions mode. Measured concentrations being again in the low pg/mL range, a large number of experiments were conducted for a better clean-up and pre-concentration of the analyte from plasma. The matrix effects were explored with the classical test of post-column infusion of the target analyte. The results obtained after injecting blank plasma processed by solid-phase extraction (SPE, on cation-exchange Isolute SCX-3 100 mg cartridges, eluted with ammonia 5% in methanol), direct protein precipitation with solvent (acetonitrile) and supported liquid-liquid extraction (Isolute SLE 400mg cartridges, eluted with methyl-tert-butyl ether) are presented in Figure 4. As expected, direct protein precipitation produced the highest ion suppression, all over the recorded chromatogram and as well in the region of the target analyte peak (retention time 2 min).

In order to evaluate the contribution of phospholipids to these matrix effects, a second experiment, precursor ion scan of m/z 184, in positive mode, over a range from 200 to 1000 Da, was performed (Figure 5). This is used to detect all phosphatidylcholines (PC), lyso-phosphatidylcholines (lyso-PC) and sphingomyelins (SM) (Jemal et al, 2010).

The precursor ion experiment on the sample processed by direct precipitation with acetonitrile shows a correlation between ion suppression on the pramipexole main transition 212.2/153.1 and the presence of PC, lyso-PC and SM (Figure 5, A and B). The extracted masses (Figure 5 C and D) confirm the presence of lyso-PC in the beginning of the chromatogram (m/z 406.5, retention time 0.6) and SM in the same elution region with the analyte (m/z 703.8, retention time 2 min). The main suppression effect between 0.2-0.4 and 1.4-1.6 minutes seems in this case

Figure 5. Ion-suppression monitored on the chromatographic traces of pramipexole (A) in the conditions of the method described in the text, after the injection of blank plasma processed by direct precipitation of plasma proteins with acetonitrile, centrifugation, dilution 1:1 with mobile phase and injection in the LC-MS/MS system. For correlation the precursor ion of m/z 184 scan (B) and extracted masses of two phospholipids (C and D) are presented below.

not produced by phospholipids; obviously salts and highly polar compounds are influencing the first region and some other lipids, amines, sterols etc could affect the second region.

Elemental sodium, potassium, iron, phosphorus and sulfur were measured in the same samples by ICP-MS (ELAN 6100 Perkin Elmer equipped with Apex-Q inlet system and PFA-ST nebulizer). Very high sodium and potassium concentrations were obviously measured in the plasma sample precipitated with acetonitrile, while in SLE the salts are not expected to be present, fact confirmed by ICP-MS (Figure 6). SPE also has high sodium levels (metal ions retained on the SCX-phase).

Similar experiments were presented in literature back in 1999, employing as model compounds caffeine and phenacetin (Bonfiglio et al). The authors have used a post-column infusion set-up and injected in the column plasma samples processed by protein precipitation with acetonitrile, liquid-liquid extraction with methyl-tert-butyl ether and SPE on Empore C2, C8, C18 and Oasis HLB. The highest ion suppression in ESI was observed for the protein precipitation, followed by Oasis SPE.

In conclusion, the sample nature, the ionization interface, mobile phase additives, stationary phases and last but most important, the sample clean-up technique, are all determining the extent of matrix effects in bioanalysis. Very interesting – and this makes the LC-MS/MS challenging but also beautiful – is that although some general rules can be established, these mechanisms are compound-dependent; in some cases there are no relevant matrix effects whilst in some others each parameter need to be optimized one by one for the best result. For our example analyte, pramipexole, positive electrospray ionization offered the best sensitivity. Different chromatographic conditions from reversed-phase at acidic or basic pH to HILIC and all three classical sample preparation methods were tested; in the end pentafluorophenyl-

propyl stationary phase was preferred, elution was performed with a mixture of acetonitrile/ ammonium formate 10mM, pH 6 (75/25, v/v) and biological sample preparation by solid-phase extraction on cation-exchange Isolute SCX-3 96-well plates has given the best recovery and less interferences.

Figure 6. Elemental content (very interesting to notice the metal ion content) determined by ICP-MS in three plasma samples processed by SLE, SPE and solvent precipitation, as described in Section 2.

3. Relevance of matrix effect in GC-MS

In gas chromatography-mass spectrometry (GC-MS) the matrix effects were generally neglected, most probably due to the sophisticated sample preparation techniques employed for GC-MS, but they are not always negligible.

Gas chromatography involves chromatographic separation of volatile and thermally stable small molecules (in certain cases derivatization is needed to induce these properties), using a gaseous mobile phase. In GC-MS the analytes eluted from the chromatographic column enter directly into the mass spectrometer source where they are ionized by bombardment with free electrons (electron impact ionization), causing the fragmentation of molecules in a reproducible way, or they are ionized interacting with a reagent gas like ammonia or methane (chemical ionization).

The application of GC-MS to biochemical analysis and especially in metabolomics is based on the pioneering work of Horning and coworkers who have demonstrated in 1971 that this technique could be used to measure different compounds present in urine and extracts. It was for the first time when the metabolic profile terminology was used (Horning et al, 1971). The next step was the diagnose of metabolic disorders by a urine test, introduced by Tanaka and coworkers (1982).

Due to the different mechanisms involved in the ionization process, there is a big difference between the matrix effects produced in LC-MS and GC-MS. While in LC-MS the co-eluted compounds affect the soft ionization mechanism in the interface, in GC-MS the ionization energy is high enough to overcome competing ionization processes, therefore mostly the GC inlet and the chromatographic column are affected by the matrix compounds, this being reflected in a high background in cases of very dirty samples or improper separation. Even though, several groups of researchers have noticed peculiar results.

In gas-chromatography the ion enhancement effect was described for the first time as "matrix induced chromatographic response" by Erney and coworkers while they were analyzing organophosphorous pesticides in extracts from milk and butterfat (Erney et al, 1993). According to their theory, during injection of standards in neat solvents, analytes could be adsorbed and thermo-degraded on the active sites of the injector or column, represented by the free silanol groups. When a real sample extract is analysed, matrix compounds block the active sites and less analyte molecules will be adsorbed, consequently enhancing their signal. In such conditions an overestimation of the calculated concentration of analytes will occur if a matrix-matched calibration curve is not used.

In the same time, the increase of the number of new active sites by gradual accumulation of non-volatile matrix compounds in the GC inlet and front part of the chromatographic column, could decrease the analyte response as "matrix – induced diminishment effect" (Hajslova et al, 2007).

Compared to LC-MS, the two phenomena of the matrix effect occur simultaneously and practically is impossible to control the formation of new active sites from deposited non volatiles matrixes. To compensate the matrix effect phenomena, the thorough clean-up of the sample to be injected (with or without derivatization) by different extraction techniques, the use of alternative calibration methods like addition of isotopically labeled internal standards or the standard addition method, as well as masking the active sites of the system by different reagents, have been adopted.

To compensate the matrix effect analyte protectants are used in GC-MS. These compounds are added both to the sample and standard solution to interact strongly with the active sites of the GC system, mainly with the silanol groups, and minimizing the matrix effect (Mastovka et al, 2005).

The evaluation of the matrix effect was used as a validation parameter for the GC-MS assay in plasma, urine, saliva and sweat of Salvinorin A, the main active ingredient of the hallucinogenic mint *Salvia divinorum*. The peak areas of extracted blank samples spiked with standards after extraction procedure were compared with the peak areas of pure diluted substances. The recovery was very good and results showed analytical signal suppression less than 10% due to co-eluting endogenous substances (Pichini et al, 2005).

4. Different approaches to minimize the matrix effect during sample preparation both in HPLC-MS and GC-MS

The techniques used for sample preparation in order to decrease the matrix effects can be grouped in five classes:

1. Non selective methods to eliminate proteins (i.e. protein precipitation)
 2. Non selective methods to separate hydrophobic compounds, generally but not always containing the analyte, from the hydrophilic fraction (i.e. liquid/liquid extraction)
 3. Selective chromatographic methods to separate off-line the analyte from the matrix (i.e. SPE)
 4. On-line chromatographic methods to separate the analyte from the matrix (i.e. column switch two-dimensions HPLC methods with different stationary phases)
 5. Analyte pre/post column derivatization to enhance analyte separation from matrix components, and ionization; this approach can be combined with any one of the previous.
1. Non selective method to eliminate proteins

It is first of all clear that these methods are useful in samples containing important amount of proteins. Protein precipitation is of modest use in normal urine samples almost free of proteins while in case of pathologic urine with high protein content it makes sense; in case of plasma there are no doubts that the protein precipitation is quite effective.

A few basic observations are important to understand the fundamentals of protein precipitation methods:

- The precipitation method used must avoid introducing a new matrix factor that we cannot separate from the analyte of interest, like heavy salt contamination;
- The precipitated samples must have a final composition adequate to guarantee the solubility of the analyte; usually, poor water soluble compounds are better precipitated in solvent then in mineral acid;

- Many precipitation methods, like those with solvents, will introduce a relevant dilution that may limit the application if the instrumental analytical sensitivity was not good enough;
- Not any precipitation conditions will bring to samples compatible with any HPLC method, like aqueous samples in case of a HILIC separation. In such cases solvent evaporation and sample reconstitution with an appropriate mobile phase is needed;
- The precipitating agent may alter, for example by chemical degradation or chemical reaction, the analyte of interest; this factor must be adequately verified;
- The precipitation process will transform relatively homogenous samples, like those of plasma, in a non-homogenous mass; adequate mixing procedures and precipitation times must be used to guarantee a complete precipitation within the all sample.

Keeping in mind the above aspects the main precipitation methods are:

- a. Solvent precipitation – Methanol, ethanol, acetonitrile and acetone, are probably the most widely employed solvents; this is also the most suitable procedure for LC/MS analysis;
 - b. Acid precipitation – Another widely used approach of precipitation based most often on halogenated organic acids like trichloroacetic acid but also on inorganic acids like perchloric acid, tungstic acid.
 - c. Salt precipitation – A less used approach in combination with LC-MS/MS exactly because of the risk of high ion suppression, but with certain established applications, e.g. zinc sulfate in immunosuppressant analysis (Koster et al, 2009)
 - d. Thermal precipitation – This is for sure the oldest method of protein precipitation but it is seldom used nowadays for analytical purposes (Fan et al, 2001). The technique remains important for protein purification.
 - e. Support assisted precipitation – similar with the solvent precipitation but using a solid phase bed (e.g. 96-well format PPT plate) that filters/retains the precipitate after centrifugation (Biotage).
2. Non selective methods to separate hydrophobic compounds from the hydrophilic fraction

In case of hydrophobic compounds, mixing the biological sample, generally aqueous, with a suitable non miscible organic solvent, will bring to a partition of the analyte in the solvent, leaving the proteins and salts in the aqueous phase. This process of liquid-liquid extraction is giving the cleanest extracts from biological matrices and it is the main sample preparation procedure for CG-MS and also widely used in HPLC-MS/MS.

The following basic procedures are used for this type of extraction:

- a. Classical liquid/liquid extraction (LLE) – In this system the aqueous based samples are mixed with an adequate solvent, shaken for a fixed period of time, allowed separating (usually by centrifugation) and the solvent is recovered and further used for analyses, after evaporation and reconstitution with an appropriate mobile phase, but some studies even optimized conditions for direct injection of hydrophobic extracts in reversed-phase conditions (Medvedovici et al, 2011)

- b. On-line liquid/liquid extraction – In this case the extraction procedure is performed by flowing the two non-miscible phases (the aqueous samples and the organic extraction solvent), generally countercurrent, in a chamber of adequate design. The organic eluent, enriched by the analyte of interest is directly analyzed without further processing, or it is evaporated and reconstituted with an appropriate mobile phase, depending on the chromatographic method.
- c. Supported liquid extraction – In this particular technique the biological samples (necessarily fluids) are absorbed over a solid support capable to retain a thin layer of liquid on its hydrophilic surface. A non-miscible solvent is then passed through the solid support containing the samples and the analyte is partitioned in the solvent; this will be recovered and further used for analysis.

The liquid-liquid extraction is also used, although currently to lesser extent, for the washing/removal of the impurities from the sample. In some cases, a series of extraction and back-extraction steps are carried out, in order to obtain a better clean-up of very dirty samples, or when the analyte suffer from high background interferences.

3. Selective chromatographic methods to separate off-line the analyte from the matrix (i.e. SPE)

Solid phase extraction techniques are widespread and very valuable sample preparation techniques. Based on the retention of the analyte on a stationary phase by different mechanisms (adsorption, ion-exchange, size-exclusion) and elution with an appropriate organic solvent at the right pH, solid-phase extraction has some advantages over liquid-liquid extraction. First of all the broad range of stationary phase beds can cover all classes of compounds, including highly polar ones; second, larger volumes of sample, even 1 L, can be loaded on the SPE cartridges, while in LLE such volumes are more difficult to handle. Of course here one must take into account that increasing pre-concentration will apply not only to the target analyte but also to the co-extracted compounds, therefore stronger matrix effects could be expected; careful optimization of SPE conditions is needed for best results.

Off-line SPE can be performed in tubular cartridges, 96-well format beds, flat disks, thin film (SPME) etc., under vacuum or applying a positive pressure from above. Solid phase micro extraction (SPME) is already routinely used in GC/MS and literature data were reviewed by Vas and Vekey (2004). SPME helps to minimize effects due to interfering organic compounds in complex matrices (Sigma Aldrich). Applications were developed for forensic, environmental or food analysis. Brown and coworkers developed and applied a SPME-GC-MS method for measuring four club drugs, gamma-hydroxybutyrate, ketamine, methamphetamine, and methylenedioxymethamphetamine, in human urine using deuterium labeled internal standards. The drugs were spiked into human urine and derivatized using pyridine and hexyl-chloroformate to make them suitable for GC-MS analysis. The SPME conditions of extraction time/temperature and desorption time/temperature were optimized to yield the highest peak area for each of the four drugs (Brown et al, 2007). Headspace solid-phase micro-extraction (HS-SPME) integrates sampling, extraction, concentration and sample introduction into a single step for GC analysis of biological fluids and materials. Compared to liquid-liquid extraction and solid phase extraction, extracts are very clean and despite the absolute recov-

eries of the analytes from whole blood are rather low, generally <10% (Mills and Walker, 2000), due to the possibility of a good pre-concentration, interesting LLOQs are achievable. The same technique was used for the determination of volatile organic compounds released by packaging expanded polystyrene by GC/MS (Kusch and Knupp, 2004).

With respect to ion suppression, for any LC-MSMS analysis is good to know whether the loss of the sensitivity is due to poor recovery or to matrix effects on the analyte ionization. A combination between an anionic – exchange SPE for sample preparation and a pre-column – analytical column switching approach was used to minimize the matrix effect and to achieve the LLOQ to 2.5 pg/ml for salmeterol in plasma (Capka and Carter, 2007).

4. On-line chromatographic methods to separate the analyte from the matrix (i.e. column switch two-dimensions HPLC methods with different stationary phases)

The isolation of the target analyte from matrix can be performed also on-line, typically with the help of a dual HPLC-system and a column-switching valve (of course more complex multiplexing systems are commercially available). On-line methods have several advantages: direct injection of the biological material (if fluid), easier for automation, therefore reducing health risks correlated with handling of hazardous material, not generating waste as used cartridges, and not the least, in a long run less expensive. As a drawback, the amount of sample loaded is limited, but the coupling with a very sensitive mass spectrometer will overcome the limited sample enrichment.

For on-line sample preparation, different stationary phases embedded as classical SPE columns, or as restricted access media (RAM) and perfusion chromatography (POROS) columns are available; another technique of interest is turbulent flow chromatography.

5. Analyte pre/post column derivatization to enhance analyte separation from matrix components, and ionization; this approach can be combined with any one of the previous.

Derivatization is commonly applied in GC/MS analysis in order to increase volatility and thermal stability of various compounds. In LC-MS/MS derivatization reactions are less used; they become important when the ionization of the target analyte is poor or matrix interferences are high. A classical example is the quantitative determination of estradiol and estrone via dansylated derivatives, in positive ionization (Nelson et al, 2004) or the determination of bisphosphonates after methylation (Tarcomnicu et al, 2007 and 2009). Because the retention time is shifted and peaks of interest elute in a region with less interferences and matrix effects, both in GC and LC-MS, derivatization is useful to improve the separation. Ionization is generally enhanced, too.

The derivatization process is carried out mostly during sample extraction or on the dried extract, but sometimes even in the injector, in case of GC-MS, or post-column in LC-MS/MS. It is done by silylation, acylation, alkylation (methylation), Schiff base formation etc.

The analyte features and sample nature (solid, fluid), amount, additives used (e.g. anticoagulant or stabilizer in case of plasma or urine) are first considerations to give an orientation in order to choose a sample preparation method. Fortunately in general it is not necessary to test

all the aforementioned techniques, but sometimes a concurrence of factors with negative impact on the results will require a step-by step approach.

A strategy to minimize the matrix effect produced by endogeneous phospholipids from plasma was developed by evaluating sample preparation and chromatographic techniques with respect to extract cleanliness, matrix effect and analyte recovery (Chambers et al, 2007). Comparisons were made between protein precipitation, liquid-liquid extraction, pure cation exchange solid-phase extraction, reverse-phase SPE and mixed mode – SPE. Two chromatographic techniques, UPLC and HPLC were used to compare resolution and matrix sensitivity. A combination of mixed-mode SPE and UPLC was proved to be the most sensitive and robust method for removing phospholipids (up to 99% relative to protein precipitation) and for determination of trace levels of drugs in plasma.

Figures 4 shows the ion suppression induced by matrix on the analyte signal; protein precipitation with solvent, SLE and SPE have been tested for the model compound, pramipexole. This example and other presented in literature (Bonfiglio, et al, 1999; Dams et al, 2003; Matuzsewski et al; 2003, Souverain et al, 2004; Mastovka et al, 2005; Pichini et al, 2005, Annesley, et al, 2007, Capiello et al, 2008) illustrate the effect of co-extracted matrix compounds on the target analyte separation and ionization. For sophisticated research, with demands of very high sensitivity and specificity, sample preparation may become very complex and need laborious optimization but the results will be rewarding.

5. How to minimize matrix effect by chromatography (HPLC and GC)

Beside sample matrix components, other potential sources of matrix effects are mobile phase impurities or additives used in HPLC (Annesley et al, 2007). For mass spectrometry pure solvents like acetonitrile or methanol are the most suitable and flow rate, applied voltage, conductivity, liquid surface tension must be properly balanced for the formation of a stable ESI spray (Chech and Enke, 2001). Higher percentage of organic solvent in the mobile phase with decreased surface tension and low boiling point will result in a more efficient desolvation of the analyte. The conductivity of the solution is also important in ESI, therefore the presence of ionic species in the solution is necessary. In positive ESI the protonated solvent clusters of methanol/water or acetonitrile/water, formic or acetic acid are ideal for facilitating the protonation. Diluted salt solutions like ammonium acetate or formate facilitate adduct formation, especially in APCI, and also improve the chromatographic peak shape. For negative ionization, diluted ammonium hydroxide is added to the aqueous solution to facilitate deprotonation (Loo et al, 1992) although formic acid, halogenated solvents (e.g. trifluoroethanol) and diluted volatile buffers (ammonium acetate or formate) are suitable as well (Chech and Enke, 2001). However protonated analytes can be observed with high pH mobile phases and deprotonated analytes under low pH conditions, as already reported (Zhou and Cook, 2000).

Ion-pairing reagents like trifluoroacetic, pentafluoropropionic, or heptafluorobutyric acids, widely used in HPLC with conventional detectors due to good retention and peak shape in the analysis of polar compounds, have known ion-suppressing effect in negative ESI; the same

for tetraalkyl ammonium hydroxide and salts in ESI positive. These additives should be carefully used in LC/MS. When the use of strong acidic ion-pair reagent is unavoidable for chromatography reasons, post-column addition of a weak acid like propionic acid could overcome the ion suppression (the so-called "TFA fix") (Apffel et al, 1995, Kuhlman et al, 1995, Annesley et al, 2007). Inorganic non-volatile buffers like phosphate and sulfate are not recommended in mass-spectrometry; they can cause salt deposits on the metal surfaces disturbing conductivity and being detrimental for ion formation and transmission (Chech and Enke, 2001).

TFA presents also matrix effects in positive ionization and an experiment confirming the well-known suppression of the ionization was described by Mallet et al. They compared the intensities of m/z 472 ion of terfenadine $[M+H]^+$, acquired in a solution containing 50/50 methanol/water, by mixing to an equivalent flow rate of 0.5% TFA and 0.5% ammonium hydroxide. In the presence of ammonium hydroxide the ion m/z 472 has shown an increase of 41% in the signal intensity compared to a decrease of 75% in the presence of TFA. With this experiment Mallet studied the influence of pH, to the ionization effects in positive and negative mode of 16 basic drugs and acidic compounds with a diversity of mass range, polarity and structure; compared to TFA formic acid and acetic acid shown less suppression effect (Mallet et al, 2004).

Benijts and coworkers have used the basic SPE procedure to study the influence of acetic acid and formic acid at two concentration levels, 0.01 and 0.1%, and of ammonium acetate and ammonium formate at the concentration 1mM and 5mM in positive and negative ionization modes, for 35 endocrine disrupting chemicals. In negative ionization mode a significant suppression of the signal was recorded at the concentration of 0.01% for both acids. The addition of buffers like ammonium formate in the mobile phase produced a slight enhancement of the ionization for all compounds excepting estradiol with a ME% of 172% (Benijts et al, 2004).

Steroids are among the compounds prone to suffer from matrix interferences both in terms of ion suppression and background interferences. We have selected here, as an example, the analysis of desogestrel from human plasma; in this case the type of ionization method was first carefully studied (as it will be presented in the next section of this chapter) and APPI (photo-spray) has given the best results in terms of sensitivity and background cleanliness (Figures 7-9). The sample clean-up was performed by SLE with a mixture of diethyl ether/tert-butyl methyl ether, which offered a good recovery and pre-concentration (obviously solvent precipitation is not suitable the molecule being highly non-polar, while SPE method needed evaporation of methanol, very time-consuming compared to the ether mixture).

Various stationary phases from different producers were tested during method development (octadecyl, octyl, pentafluorophenylpropyl, phenyl). Figure 8 presents the chromatograms recorded on the transitions selected for desogestrel after the injection of blank plasma spiked with analyte at 1 ng/mL, clean-up by SLE. Separation from the interfering matrix peak in the vicinity on the octadecyl column (Eternity C18 10 cmx2.1 mm, 2.7 μ m) was not satisfactory at first; playing on the gradient a cleaner delimitation of desogestrel peak was achieved.

Figure 7. Chromatograms recorded on two transitions selected for desogestrel (A, C: 293.3/133.2 and B, D: 293.3/197.2) in APPI ionization after the injection of pure methanol (A, B) or Desogestrel solution at 1 ng/ml in water/methanol (1:1, v/v). Desogestrel elutes at 4.8 min. As it can be observed each transition is differently affected by matrix components. Column: HSF5 10 cmx2.1 mm, 5 μ m (Supelco); Mobile phase: aqueous formic acid 0.1% and acetonitrile; Flow: 0.3 mL/min; gradient elution; injection volume: 30 μ L In the next step, chromatography was optimized further for minimizing matrix effects.

Figure 9 shows an overlay plot of two extracted plasma samples, a blank and a spiked concentration (1 ng/mL), in the new chromatographic conditions. Desogestrel peak is clearly visible, as indicated by the arrow. Good results were obtained with the phenyl stationary phase as well.

A huge selection of stationary phases and mobile phases is available at the moment and their right combination can make significant improvements in the chromatographic separation, thus in diminishing matrix effects. As already mentioned before, sample preparation must be also seen in view of the chromatographic technique selected.

As it can be observed in Figure 7, on the chromatographic traces selected for desogestrel several quite intense peaks were recorded even after injecting pure methanol.

No miraculous HPLC or GC separation method permit to escape of matrix effects but a few general considerations are reported next and they may serve as a guideline to improve the analytical work minimizing matrix effect:

Figure 8. Chromatograms recorded on two transitions selected for desogestrel (293.3/133.2 and 293.3/197.2) in APPI ionization. Desogestrel spiked at 1 ng/mL in plasma, extracted by SLE. Column: Eternity C18 10 cmx2.1 mm, 2.7 μ m (Akzo Nobel); Mobile phase: aqueous formic acid 0.1% and acetonitrile; Flow: 0.2 mL/min; gradient elution; injection volume: 30 μ L. Desogestrel eluted at 4.94 min.

1. Void peak and source contamination – As aforementioned, salts, peptides and other polar compounds generally not retained in the chromatographic column are important matrix factors. These compounds tend also to deposit on the ionization sources extending the matrix effects far beyond the elution times, often accumulating from one injection to the following. It is nonetheless very useful and simple introducing in the chromatographic system, both in case of HPLC and GC, of a diverter valve (controlled by the computer system or the HPLC pump) to send to waste the initial chromatographic peak. This approach will avoid heavy source contamination improving the system stability. In case of GC separations, conventional valves can be used but other interesting alternative are fluidic switches without moving parts and no risks of introducing cold spots in the chromatographic system and/or deteriorate the peak shape;
2. Fast separations are good but if the resolution is maintained – It is always convenient to get faster methods but it is important to avoid inadequate separation in order to be quick; the matrix peaks must be adequately isolated from the peaks of interest.
3. LC x LC or GC x GC methods – It is clear that two dimension separations permit to get the maximum in term of isolation of the compound to be analyzed from matrix peaks. In case of difficult analyses it is always difficult to evaluate if a complex sample preparation is convenient and more effective than a better HPLC/GC separation, in principle both approaches must be each time evaluated. Experiments carried out by Pascoe and co-workers are a good example; the authors tested a series of stationary phases with a column-switching set-up and reported a reduction in matrix effects (Pascoe et al, 2001).

As a general rule, the use of stationary phases with different retention mechanisms (i.e. ion exchange and reversed phase or hydrophobic with polar GC columns) is the most effective

Figure 9. APPI ionization. Desogestrel - An overlay of blank plasma extract and plasma spiked with desogestrel at 1 ng/mL. Chromatograms recorded on the transition 293.3/197.2, in APPI ionization. Column: Eternity C18 10 cmx2.1 mm, 2.7 μ m (Akzo Nobel); Mobile phase: aqueous formic acid 0.1% and acetonitrile; Flow: 0.2 mL/min; gradient elution; injection volume: 30 μ L. Desogestrel peak is indicated by the arrow.

combination to maximize the separation of analytes from matrix and, at the same time, to increase the selectivity.

4. Column overload and source overload – A common trend is to increase the loop size when the sensitivity is inadequate considering that more analyte in the source is increasing the chromatographic peak; this fact is often wrong for two important reasons. First, increasing the injection volume may bring to a column overload with modification of peak shape and a peak normally not affected by matrix can become disturbed by it due to a broadening of the matrix peak. A deterioration of peak shape is often observed with large injection volumes making no advantage in terms of S/N ratio improvement. As a paradox, in complex matrices, in case of inadequate sensitivity it is often interesting to test the injection of a more diluted sample to understand if the low sensitivity is really due to inadequate amount of sample or an excessive matrix effect.
5. Mobile phase composition – As discussed above, in HPLC it is always important to remember that different mobile phases may present quite different matrix results; the same also for the type of MS ionization (see next section). It is therefore important to test several mobile phases and ionization conditions in order to minimize the matrix effects.

6. Flow rate – Using lower flow rate is a big advantage in order to minimize matrix effects. The ionization efficiency improves significantly with lower flow rate, less contaminants are introduced in the source contributing to keep the ion optics cleaner, higher content of water (this means often better separation) in the mobile phase can be handled without too much loss in sensitivity and less heating is needed in the source often resulting in a less important chemical background, partially responsible of matrix effects.
7. Stationary phases – Evidently, it is not possible to review all existing columns and to suggest special kinds because each analyte has its own properties and such detailed presentation is outside the scope of this chapter. It is however interesting to summarize a few key points in order to minimize matrix effects and get the maximum of results. First, when developing a new analytical method it is important to consider the polarity of the analyte and, in comparison, the expected type of matrix present in the sample. As an example in urine one will not have problems with proteins while a high salt content (e.g. biliary salts) and other polar compounds will dominate the matrix. In such conditions, for the analysis of highly polar compounds, it can be interesting to consider ion exchange columns or HILIC chromatography, instead of classical reversed phase columns. In case of non-polar compounds in plasma, matrix effects from phospholipids are critical and these endogenous products are also quite apolar creating peculiar matrix problems. In such cases a careful choice of a column able to retain differentially the analyte is important; columns like phenyl or pentafluorophenylpropyl can be quite selective in retaining the analyte if it has an aromatic group normally absent in phospholipids. The possibilities are endless but the problem must be evaluated before screening blindly a large number of stationary phases.
8. Analyte derivatization – Derivatization methods, despite not being strictly chromatographic methods can often bring to results otherwise impossible, especially when the derivatization changes the analyte polarity. Several examples are available where highly polar compounds, like aminoacids, biphosphonate, catecholamines, aminoglycosides, can be transformed by derivatization in less polar compounds easily separated by GC or HPLC.
9. High resolution mass spectrometry can be very useful for the analysis of dirty biological extracts, through a better separation of the analyte from background interferences. Ultra-high pressure liquid chromatography (UHPLC), micro, capillary and nano-LC provide high resolution separations (increased number of theoretical plates) with very narrow peaks thus easing the possibility of changing the analyte retention time towards regions in the chromatogram less affected by matrix. Many labs are transferring their methods now towards ultra-high-pressure chromatography (UHPLC); matrix effects have been evaluated and improvement reported (Van de Steene and Lambert, 2008).
10. HPLC column, solvents, plastic and polymer residues, reagents as source of matrix – Never forget that column bleeding both in GC and HPLC can be an important cause of matrix effect. In case of poor chromatographic sensitivity with compounds otherwise ionizing properly it is useful to check different columns, also within the same type of bonding, for matrix effects. Unfortunately similar problems may come also from solvents, water and

salts employed in HPLC, as well as from plastic and polymer residues from tubes, 96-well plates, caps and lids, filters, SPE beds, etc. (Mei et al, 2003; Capiello et al, 2008).

6. Optimization of MS interfaces and ionization conditions to minimize matrix effect

The first point to consider is the choice of interface type. In this respect it is important to observe that matrix effects are more evident in conditions of poor ionization, therefore generally the source with the best ion efficiency is the first choice. A second point to consider is that matrix effects also derive from a competition between matrix ions and analyte ions at the level of ion sampling in the orifice area. Clearly, a source giving minimum ionization efficiency for the matrix is also effective in minimize matrix effects over the analyte ionization; this fact can be well appreciated with sources having specific ionization mechanisms, like the atmospheric pressure photoionization source (APPI), that may give interesting advantages in terms of matrix effects. However, only experimental tests will confirm and help to define the most appropriate ionization interface.

Once defined the source to be used an important step is the definition of the ionization polarity. In this respect the chemical structure of the analyte may impose a choice but it is also important to consider the restriction coming from the mobile phase composition: one will never get a reasonable negative ionization in presence of trifluoroacetic acid, while formic or acetic acids are fine; no chance to work in positive mode with a strong base like tetrapropylammonium in the mobile phase but diluted ammonium hydroxide is good.

The aspects of mobile phase composition and ionization mode being clarified, an important stage in the source optimization is not only to play on the best signal for the analyte but also to look for the lowest background ionization. It is in fact important to find the situation where the ratio between background ions and analyte is the most convenient. Ion transfer voltage (ESI, APPI) or needle current (in case of APCI), declustering (orifice) voltage, nebulization conditions (temperature, gas flow rates) and source position optimization (depending upon the kind of source) are some of the key elements of optimization aiming to improve this ionization ratio.

In our example, we have optimized the ionization interface for the analysis of desogestrel. Due to the high background in ESI on the most intense multiple reaction monitoring (MRM) transitions corresponding to the analyte, several other less intense transitions were explored under selected chromatographic conditions (column HSF5 10 cmx2.1 mm, 5 μ m, mobile phase: aqueous formic acid 0.1% and acetonitrile in gradient elution at 0.3 mL/min, mass spectrometer: API 5000 triple quadrupole). The result is presented in Figure 10. Further development included testing of APCI (Figure 10) and APPI (Figures 7-9) ionization interfaces.

Photoionization can give excellent results in terms of ionization efficiency for aromatic compounds or structures with multiple conjugated double bonds (Yang and Henion, 2002, Tiedong 2004, Yamamoto 2006) and proved to be the best also for our target compound,

Figure 10. Chromatograms recorded on two transitions selected for desogestrel (293.2/91.2 and 293.2/115.2) in ESI ionization (A, B) and APCI ionization (C, D). Desogestrel – 10ng/mL standard in water/methanol (1:1, v/v). Column: HSF5 10 cmx2.1 mm, 5 μ m (Supelco); Mobile phase: aqueous formic acid 0.1% and acetonitrile; Flow: 0.3 mL/min; injection volume: 30 μ L. The same gradient was used in both ionization modes; the difference in retention time (3.69 vs. 4.17 min) resulted because the acquisition was 0.5 min later triggered in chromatograms A and B.

although matrix issues were not completely solved. APPI was selected for the final method; chromatography experiments were presented in *Section 5*.

As demonstrated by Mei and coworkers, matrix effects are also not only ionization mode dependent but also source-design dependent (Mei et al, 2003). They have injected plasma processed by solvent precipitation, using identical LC set-up, into three instruments from different manufacturers, equipped with ESI as well as with APCI interfaces. The measurements were performed in positive ions mode, monitoring 8 MRM transitions, chromatographic separation employing a Metachem Basic 4.6x50 mm, 5 μ m column eluted in gradient with ammonium acetate 10mM containing 0.005% acetic acid and methanol. For the Micromass Quattro tandem mass spectrometer, Mei et al found that APCI source is more sensitive to matrix effects in the studied conditions. Overall, 22 examples of matrix effects were identified across various regions of the chromatographic gradient; most of these involved early-eluting polar compounds. One of the monitored molecules showed ionization enhancement in presence of Li-heparin as anticoagulant.

Capiello and coworkers have studied as an alternative to ESI an efficient LC-MS interface based on direct electron ionization (Direct-EI) for the analysis of small and medium molecular mass compounds (Capiello et al, 2008). They have quantitatively evaluated the impact of matrix effects on this type of ionization, using for experiments plasma or river water samples. Phenacetin and ibuprofen were used as model compounds. Plasma samples were extracted by LLE or SPE; water samples by SPE. The majority of matrix effects observed in LC-ESI-MS were surmounted using the LC-Direct EI-MS interface. There is to mention though that in this

case also the LC set-up was different, respectively a nano-LC system was used in combination with Direct-ESI; nano-LC itself brings improvement in overcoming matrix effects also when ESI is employed. (More on this topic in *Section 8*)

7. Accepting matrix effects as unavoidable in analyses of real samples; approaches to obtain reliable quantitative results

As a conclusion of the discussion so far, there is no doubt that both in quantitative and qualitative bioanalysis, matrix effects are present. These effects are unseen in the chromatogram but can have deleterious impact on methods accuracy and sensitivity; it is important that they are identified and addressed in method development, validation, and routine use of HPLC–ESI–MS/MS (Taylor, 2009, Hall et al, 2012).

Adequate measures must be taken to guarantee that results are reliable; these actions can be divided in two groups:

1. Identification of the relevance of matrix effect in the analytical conditions used
2. Introduction of corrective factors to compensate the unavoidable matrix effects inherent to the analytical method employed.

First kind of actions groups the procedures used to detect and/or quantify the matrix effects present in an analytical procedure. The first method was proposed by Bonfiglio et al (1999) and it is based on the continuous infusion of the compound to be analyzed in the mass spectrometer equipped with the selected ionization sources. Just before entering in the source, this is mixed with the mobile phase from the HPLC pump to be used for the analytical procedure. Blank matrix samples extracted using certain procedure are injected in this system, with or without chromatographic column. A few examples of this method were presented in Figure 4 (*Section 2*). As it can be seen this approach allows very well to test different HPLC procedures, especially in order to improve separation conditions, trying to avoid the co-elution of the analytes of interest with peaks having an important matrix effect. Weak points of this approach are its complexity, the difficulty to quantitatively define the impact of the matrix effect and the risk to contaminate the interface with high amount of analyte through infusion.

In order to overcome this fact, the alternative approach was proposed by Buhrman et al (1996) then by Matuszewski et al (2003). In such method extracted blank samples (representative of matrix and analytical procedures to be tested) are spiked with a known amount of the analyte and the results are compared to the ones obtained analyzing the same compound at the same concentration dissolved in mobile phase. Ratios between these data are now employed and recommended from several regulatory authorities as a quantitative “matrix factor”, with well-defined limits of acceptance (Viswanathan et al, 2007).

Considering the corrective actions, in order to compensate the matrix factor, the use of internal standards (in particular analogue of the analyte labeled with stable isotopes) is definitively the main approach to solve the problem (Tranfo et al, 2007). In case of other chemically related

analogues, normally used in HPLC with UV or fluorescence detectors to correct for extraction and/or injection variation, their matrix factor in LC-MS can be quite different from that of the analyte; in such cases a verification of the matrix factor for analyte and as well for the internal standard is useful even if they eluted in the same retention time with the analytes. When the internal standard is not co-eluting with the analyte, the influence of interfering compounds on the ionization can be different thus the quantitative results could be biased. It is noteworthy that also in case of stable isotope labeled internal standards significant differences of retention time, versus the non labeled compound, can be observed sometimes (especially when the mass difference is high, e.g. d7- or d9-labeled molecules, or in case of HILIC separations), making critical the matrix effect correction if a sharply eluting peak of an interfering compound is present. Due to this fact different labeling, like ^{13}C , could be used instead of the more commonly employed deuterium to minimize the chromatographic shift.

In case an internal standard cannot be used or it is not available blank samples spiked with the analyte of interest must be always analyzed in parallel to be sure that the analyte is not influenced by matrix avoiding unreliable results. A spiking of a known analyte concentration on the same sample to be analyzed is also an interesting approach (if the sample amount is enough) to guarantee the appropriateness of the measurement performed.

8. Future perspectives

After so many evidences of the relevance of matrix effects in bioanalytics what can we expect next? Do we have possibilities to further improve this situation?

In the next we will consider the main three areas explored in this paper and the chances of development for the future:

1. *Sample preparation* – This area knows continuous improvements; more and more selective extraction methods provide cleaner sample extracts, with reduced matrix content. In this context the development of better immunopurification media (more chemically stable, easier on-line applications) for an always larger palette of antigens, the appearance of newer molecular imprinted polymer (MIP) columns for specific chemical groups and the possibility to do automated solid-phase micro-extraction (SPME) processing large number of samples at the same time are between the most attractive opportunities. SPME seems to be potentially very interesting, its simplicity minimizing liquid handling, the possibility for reusing the sorbent by adequate washing (much simpler than in SPE), the possibility to introduce immunopurification media or MIP, and finally the potential for down scaling to the micro level are between the most intriguing aspects.
2. *HPLC methods* – The choice of stationary phases, with enhanced separation properties, is constantly growing, and one of the directions with a lot of potential is currently hydrophilic interaction liquid chromatography (HILIC), with increasing number of applications in bioanalysis fields (Hsieh, 2008, Van Nuijs et al, 2011).

However the improvement in equipment seems to be the most interesting part. Years are passed from the time when LC/MS producers were struggling to get higher flow rate sources pushed by customers acquainted to large HPLC columns and unsatisfied by the technical performance of micro-column on micro-HPLC system. UHPLC is nowadays widespread and better results in terms of matrix effects compared with classical HPLC were already reported (Novakova et al, 2006, Van de Steene et al, 2008).

Micro, capillary, nano-HPLC columns are now easily available, robust, reliable and performing very well in terms of separation. All this also thanks to better HPLC systems, permitting to exploit adequately these columns. It is well recognized that matrix effects are reduced at lower flow rates, with a concomitant increase in term of sensitivity; it has to be seen if a revolution will take place in LC/MS as it happened in GC-MS years ago when going from packed to capillary GC columns. A lot of improvement will come for sure passing to packed columns in the sub millimeter diameter range and below, eluted with very low flow rate. Experiments performed recently in our laboratory with a 0.3 mm inner diameter column were very promising. An example is presented in Figure 11.

Figure 11. Chromatograms recorded on the MRM transitions of Diosmetin-3,7-O-Glucuronide (A, C - 653.222/301.1) and Diosmetin-7-O-Glucuronide/ Diosmetin-3-O-Glucuronide (B, D - 477.237/301.1) after the injection of extracted plasma samples spiked at 0.1 ng/mL (A, B) or 15 ng/mL (C, D); elution at 50 μ L/min on Halo C18 (0.3x50 mm, 2.7 μ m, 90A packing – Eksigent) column. Diosmetin-7-O-Glucuronide – retention time 2.88 min; Diosmetin-3-O-Glucuronide – retention time 2.81 min

Diosmetin is a metabolite of diosmin, a natural flavonoid found in most fruits and vegetables; moreover these contain a series of compounds with the same mass and related structure giving numerous interferences, therefore on conventional LC columns the separation was not possible below certain concentrations. Figure 11 presents the chromatograms recorded using a sub-

millimeter column, Halo C18 (0.3x50 mm, 2.7 μ m, 90A packing – Eksigent) eluted at 50 μ L/min in gradient with a mobile phase containing water+0.5% formic acid and acetonitrile with 0.5% formic acid. Plasma spiked at 0.1 ng/mL or 15 ng/mL was injected. As it can be noticed, five peaks were distinctly separated in the biological extract within an interval of 0.25 min; in these conditions it was possible to obtain a blank sample from patients with special diet. This powerful separation helps in reducing matrix effects and benefits also from the advantage of very low flow-rate.

The hyphenation of separation techniques like isoelectrophoresis/capillary electrophoresis and HPLC is another area not yet well exploited but offering a lot of potential to get cleaner samples with minimal matrix effects.

3. *MS Ionization interfaces* – An exhaustive presentation is not possible in this area; however a few examples of potential new ways to reduce matrix can be introduced.

In the last years ion mobility become more and more present in the MS analytic instrumentation range. In particular ion mobility (IM) techniques have created a possibility to play on the gas phase in front of the sampling orifice of the mass spectrometers, selecting the relevant ions to be analyzed. These applications are quite at the beginning and the real impact on the matrix effects has not been fully explored, until now the focus being more on the enhancement of the analytical selectivity. The difference in cleaning the matrix interferences can be impressive, as it can be seen in the example of clenbuterol analysis from human urine (Figure 12) without further processing; the sample is just diluted 1:1 and injected in the LC-MS system (AB Sciex).

Figure 12. Clenbuterol Spiked in Human Urine (diluted 1:1 prior to analysis). QTRAP[®] 5500 vs 5500 with SelexION[™] Technology. (Reproduced with the permission of AB Sciex).

Other groups are also focusing on very low molecular mass ions analysis, that most often are considered background ions, hence optimizing instruments for liquid or gaseous matrices. An

Figure 13. Analysis of the Lithium isotopes at a concentration of 10^{-8} mol/L using an instrument specially designed for low molecular mass compounds. (Reproduced with the permission of AMD Intectra).

API Interface with ESI/APCI Glow Discharge on a double beam magnetic sector was developed by AMD; the interface can switch between LC, GC or CE inlet without needs of any system modification. The high-resolution results (Figure 13) obtained in the low mass range (like alkali metals from m/z 6 to 39), generally affected by huge interferences of artifacts, are very interesting (AMD Intectra GmbH).

Most probably exciting results will come next from this kind of sources in combination with newer techniques of ion sampling from the atmospheric pressure side to the high vacuum chamber. We are going from orifice – skimmer sources always more to ion guide systems (with small quadrupoles or lens cascade) permitting to obtain a higher transmission and improving the separation from neutral molecules, solvent clusters and allowing a cut-off based on ion characteristics.

This brief example of future progress in ion sources wants to be just a message on how much the hardware development remains open for important improvement in the matrix effects control.

To conclude let's hope that new developments will be so impressive to make matrix effects something of the past and all problems presented in this chapter just scientific curiosity. Who knows?

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