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Evaluation of the influence of protein precipitation prior to on-line SPE–LC–API/MS procedures using multivariate data analysis☆

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Abstract

Matrix effects on mass spectrometry (MS) response were investigated with three atmospheric pressure ionization (API) sources after on-line solid-phase extraction (SPE) of human plasma. On-line SPE was evaluated with one restricted access material (RAM), two large particle supports (LPS) and one monolith. A sample protein precipitation (PP) with acetonitrile (2:1) and a direct injection were tested. Principal component analysis (PCA) was performed to simplify data presentation and interpretation. Protein precipitation was found to be mandatory for reducing signal modification. Regarding sensitivity towards matrix effects after PP, atmospheric pressure photoionization (APPI) was globally the least sensitive ionization mode while electrospray ionization ESI was the most sensitive.

Keywords: Matrix effects; APPI; Human plasma; On-line SPE; Post-column infusion

1. Introduction

Over the past decade, atmospheric pressure ionization (API) sources have been widely used to perform efficient mass spectrometry coupled to liquid chromatography (LC–MS). Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) sources have become the classical interfaces for hyphenating LC to MS in order to perform fast, selective and sensitive analysis of pharmaceuticals in biological fluids [1–6]. Both sources are soft ionization techniques leading to protonated or de-protonated species without important fragmentation and have found many applications in the analysis of polar, moderately polar and low polarity analytes in solution. Atmospheric pressure photoionization (APPI) is the latest source among API techniques which extends the field of application of LC–MS to apolar molecules. The photoionization (PI) process was already used about 30 years ago as a detection method (PID) in gas chromatography [7–12] and coupling LC to PI was performed by Locke et al. [13], and later by Driscoll et al. [14]. Moreover, the first applications of PI as an ionization process for MS analyses were performed in the 1980s by Revel'skii et al. [15]. Finally, the use of APPI to carry out LC–MS analyses was described in 2000 [16,17], and as already reported for ESI and APCI, the source design has a tremendous effect on the ionization process [18].

In the literature, it has already been shown that complex biological matrices, such as urine, saliva, plasma, serum and whole blood could alter the response of an analyte when LC–MS analyses are performed without adequate sample preparation and/or good chromatography. This phenomenon, called the matrix effect, is due to co-elution of endogenous compounds, such as proteins, lipids, sugars or salts interfering with the analytes during the ionization process. Matrix effects have already been widely reported, especially for ESI and APCI [18–34], while APPI has been investigated to a lesser extent [35–41]. Different approaches are described to investigate matrix effects, and the most currently implemented technique uses the continuous post-column infusion of an analyte solution [19,21,22,26,27,31,32,34,42–46]. In ESI, the suppression mechanism has been explained through different models [23,47], but it is generally accepted that an ionization competition occurs within the different eluted compounds. APCI and

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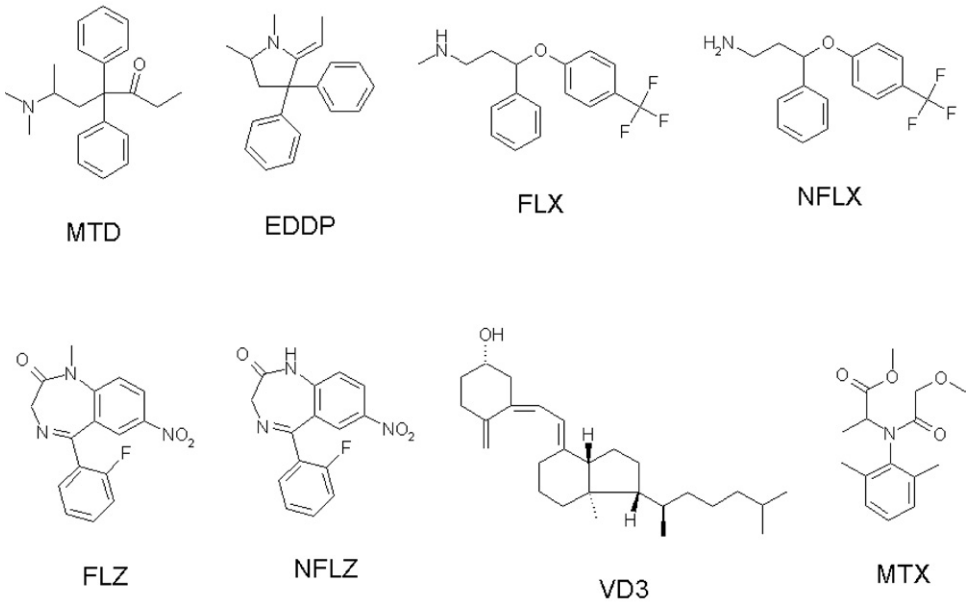

Fig. 1. Chemical structures of model compounds.

APPI are often reported to be less sensitive to such effects than ESI, because the ionization process takes place in the gaseous phase [18,33,43,48].

In order to overcome signal modification when complex matrices are analyzed by LC–MS, sample clean-up procedures must be operated to remove potential interfering substances. For that purpose, off-line and on-line sample preparation techniques can be employed. The most commonly used techniques among off-line procedures are liquid–liquid extraction (LLE), solid-phase extraction (SPE) and protein precipitation (PP) [49,50]. They can be operated manually as well as automatically using robots [51] as well as on cartridges and well-plate formats [52–54].

On-line sample preparation procedures coupled to LC–MS *via* a column-switching approach for the analysis of drugs in biological matrices have already been widely described elsewhere [55,56]. More particularly, one of the authors has studied matrix effects in LC–ESI/MS and LC–APCI/MS with off-line and on-line extraction procedures [57]. With methadone selected as a model compound, APCI was less susceptible to ion suppression regardless of the sample preparation procedure.

The aim of this paper was to further extend the study of matrix effects with on-line SPE–LC–API/MS with other compounds and to determine more particularly the protein precipitation impact applied before on-line extraction. Thus, matrix effects were evaluated with ESI, APCI, and APPI sources coupled to a single quadrupole mass spectrometer. Four commercially available extraction supports (one restricted access material (RAM), two large particle supports (LPS) and one monolith (MNL)) were used in the column-switching configuration. Direct injection of plasma without (sample dilution) and with a sample pre-treatment (protein precipitation with acetonitrile) were performed and compared. Each extraction support was investigated with pharmaceutical compounds selected as model compounds and their primary metabolites, namely methadone (MTD), 2-ethylidene-1,5-dimethyl-1,3-diphenylpyrrolidine (EDDP), fluoxetine (FLX), norfluoxetine (NFLX), flunitrazepam (FLZ) and norflunitrazepam (NFLZ), as well as vitamin D3 (VD3) and an apolar pesticide, metalaxyl (MTX) (Fig. 1).

2. Experimental

2.1. Chemicals

Methadone hydrochloride (MTD) was purchased from Sintetica (Mendrisio, Switzerland) and 2-ethylidene-1,5-dimethyl-1,3-diphenylpyrrolidine perchlorate (EDDP) was from Cerilliant (Austin, Texas, USA). Fluoxetine (FLX) was provided by Heumann Pharma (Nuremberg, Germany) and norfluoxetine (NFLX) and perchloric acid were from Sigma (Steinheim, Switzerland). Flunitrazepam (FLZ) was purchased from Hoffmann-La Roche Ltd. (Basel, Switzerland), and norflunitrazepam (NFLZ) was kindly provided by Dr. C. Staub of the Institut Universitaire de Médecine Légale (Geneva, Switzerland). Vitamin D3 (VD3) and acetone (ACT) were purchased from Fluka. Metalaxyl (MTX) was kindly provided as a standard solution by the Service de Protection de la Consommation (Geneva, Switzerland). Structures of the molecules are reported in Fig. 1. Acetonitrile (ACN), methanol (MeOH) and formic acid 98% were purchased from Panreac (Barcelona, Spain) and water was provided by a Milli-Q Gradient A10 water purifier system from Millipore (Bedford, MA, USA). Human blank plasma with sodium citrate was obtained from Laboratoire de Sérologie Transfusionnelle des Hôpitaux Universitaires de Genève (Geneva, Switzerland). All chemicals were of the highest purity grade commercially available and all reagents used were of HPLC grade.

Stock solutions of MTD, EDDP, FLX, NFLX, FLZ, NFLZ and MTX were prepared in a mixture of water/ACN (1:1, v/v) at a concentration of 1000 μg mL^{-1} each. Stock solution of VD3 was prepared in methanol at a concentration of 1000 μg mL^{-1}. For the optimization of the ion source parameters, standard solutions at a concentration of 10 μg mL^{-1} were prepared by dilution of stock solutions in the mobile phase. For post-column infusion, a solution containing the eight drugs at 2 μg mL^{-1} was prepared by dilution of stock solutions in the mobile phase. Blank plasma was stored at −22 °C and then defrosted at room temperature for 1 h.

2.2. Instrumentation

All experiments were performed on an Agilent Series 1100 LC system (Agilent Technologies, Waldbronn, Germany) equipped with an autosampler, a binary pump and a six-port switching valve. An additional Agilent Series 1100 LC isocratic pump was included in the system for the column-switching configuration. The LC system was coupled to an Agilent Series 1100 UV-detector and an Agilent Series 1100 MSD single quadrupole equipped with orthogonal ESI, APCI or APPI sources. MS parameters were optimized for each ionization source and are reported in Table 1. Nitrogen was used both as a nebulizing and a drying gas. MS detection of protonated FLX, NFLX, MTD, EDDP, FLZ, NFLZ, MTX and VD3 was conducted with each source in the single ion monitoring mode (SIM) at 310, 296, 310, 278, 314, 300, 280 and 385 Th, respectively, with optimized skimmer voltages (Table 1). The Chemstation software suite A.09.03 (Agilent Technologies) was used for instrument control, data acquisition and data handling. Post-column infusion was achieved by a Harvard 11 Plus Single Syringe pump (South Natick, MA, USA).

2.3. Sample handling

2.3.1. Protein precipitation with acetonitrile [50]

Five hundred microlitres of blank plasma was added to 1000 μL of acetonitrile, vortex mixed and centrifuged for 5 min at 6000 × *g*. The supernatant was collected and transferred into a vial.

Table 1
Optimized parameters for ESI, APCI and APPI sources

	ESI parameters	APCI parameters	APPI parameters
Nebulization pressure (psi)	25	10	35
Drying gas flow rate (L min^{-1})	11	5	7
Drying gas temperature of (°C)	300	350	300
Capillary voltage (V)	3000	3500	2000
Vaporizer temperature (°C)	–	350	250
Corona discharge (μA)	–	3	–
MTD, FLX skimmer voltages	100	100	100
EDDP skimmer voltages	140	140	140
NFLX skimmer voltages	80	80	80
FLZ skimmer voltages	150	150	150
NFLZ skimmer voltages	130	130	130
VD3 skimmer voltages	120	120	120
MTX skimmer voltages	100	100	100

2.3.2. Protein precipitation with perchloric acid/acetonitrile

Five hundred microlitres of blank plasma was added to 1000 μL of a 12% perchloric acid/acetonitrile (60:40) mixture, vortex mixed and centrifuged for 5 min at 6000 × *g*. The supernatant was collected and transferred into a vial.

2.3.3. Dilution with water (direct injection)

Seven hundred fifty microlitres of blank plasma was added to 750 μL of water, vortex mixed and centrifuged for 5 min at 6000 × *g*. The supernatant was collected and transferred into a vial.

2.4. On-line post-column infusion configuration

The on-line column-switching and post-column infusion setup is shown in Fig. 2 as already reported elsewhere [32]. A chromatographic Purospher STAR RP-18e column (55 mm × 2.0 mm i.d., dp 3 μm) from Merck (Darmstad, Germany) was used for chromatographic separations. Selected extraction supports, coupled on-line with the analytical column, were as follows: an Oasis HLB (20 mm × 2.1 mm i.d., dp 25 μm) from Waters Corporation (MA, USA), a LiChrospher RP-4 ADS (25 mm × 2.0 mm i.d., dp 25 μm) from Merck (Darmstadt, Germany), a Cyclone Turboflow HTLC (50 mm × 1.0 mm i.d., dp 50 μm) from Cohesive Technologies (MA, USA) and a Chromolith Flash (25 mm × 4.6 mm i.d.) from Merck.

The mobile phase for performing the elution of extracted compounds in the back-flush mode was constituted of 0.1% (v/v) formic acid in water:ACN (65:35) (v/v) for ESI and APCI (MP1) and 0.1% (v/v) formic acid in water:ACN:acetone (65:35:10) (v/v/v) for APPI (MP2), both delivered at a flow rate of 300 μL min^{-1}. A 2 μg mL^{-1} drug cocktail solution was post-column infused with the syringe pump at a flow rate of 2 μL min^{-1}.

Fifty microlitres of mobile phase (MP1 or MP2), precipitated blank plasma, and diluted blank plasma were injected into the extraction supports with a loading mobile phase (MP3) constituted of 0.1% (v/v) formic acid in water:ACN (95:5) (v/v). The sample loading was performed in all cases at 4 mL min^{-1}. For the LiChrospher RP-4 ADS extraction support, samples were also loaded at 0.8 mL min^{-1}. After simulating the transfer step of the extracted compounds from the pre-column toward the analytical column, the valve was set to its starting position for reconditioning the extraction support with a mobile phase containing 0.1% (v/v) formic acid in water:ACN (20:80) (v/v) (MP4). Table 2 shows the switching times for each extraction support.

2.5. Data handling software

Data handling (principal component analysis and hierarchical cluster analysis) was performed with the XLStat 6.5 (AddinSoft, France) software package.

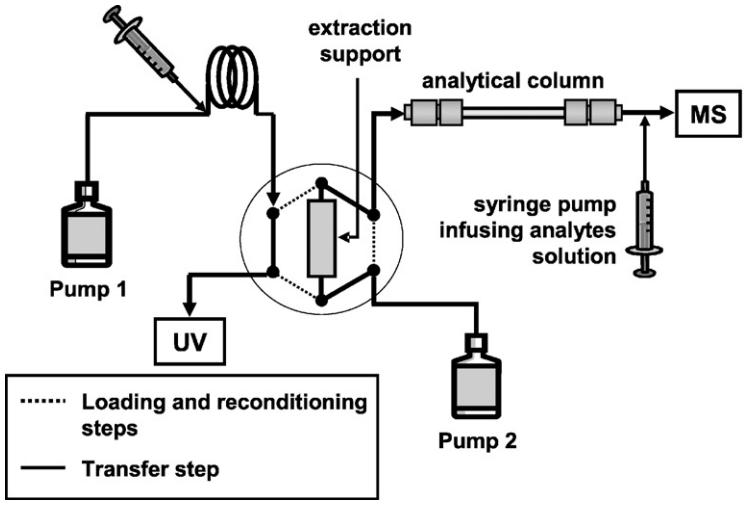

Fig. 2. Column-switching configuration with post-column infusion system.

3. Results and discussion

3.1. Used strategy

Different samples were injected in all extraction supports in the column-switching configuration: loading mobile phase, blank plasma diluted 1:1 with water (direct injection) and blank plasma precipitated 2:1 with ACN. A blank plasma precipitated 2:1 with a 12% $HClO_4$/ACN (60:40) mixture was tested for protein precipitation but results were unsatisfactory in terms of sample stability (turbidity of the solution increased as a function of time). Therefore, this procedure was not selected in this study. Each plasma sample was injected in triplicate alternatively with a mobile phase injection and LC–MS data were recorded with the three API sources. The loading and eluting mobile phase composition and flow rate were chosen on the basis of our previous work [32]. The loading flow rate was fixed at 4.0 mL min^{-1} owing to experimental setup limits considered for LPS and monolith supports. The LiChrospher RP-4 ADS extraction support was tested at conventional (0.8 mL min^{-1}) and rapid flow rates (4.0 mL min^{-1}). Indeed, and as already reported in the literature [58], this material is made of 25 μm diameter particles allowing the application of a relatively high flow rate without excessive back-pressure.

Regarding MS conditions, parameters were optimized for each ion as a function of the source and the best compromise was selected (see Table 1). Acetone was chosen as the APPI dopant for several reasons. It presents superior solubility in aqueous phases, low toxicity versus toluene and is well adapted to basic compounds [16]. In all experiments, the dopant was directly added to the eluting mobile phase. For all samples injected in triplicate, the same profiles were obtained. Therefore, for the sake of clarity, only the last profile was selected and used for further interpretation. An example of the total ion current (TIC) profile with the Chromolith Flash extraction support is given in Fig. 3.

To study the ionization modifications of each compound separately as a function of the sample pre-treatment, the nature of the extraction support and the API source, extracted ion currents (XIC) were used for the data treatment. Signal disturbances observed after injection of precipitated plasma and the mobile phase were similar. They were mainly due to the column-switching setup and the baseline was rapidly stabilized. To obtain signals regarding matrix effects only, the mobile phase injection signal was subtracted from the diluted and precipitated plasma injections. Therefore, the investigated MS signal modifications were due solely to endogenous material without external effects due to the column-switching setup.

Matrix effects were characterized in terms of signal alterations, namely the relative enhancement or suppression signal intensity and the time window. The relative signal intensity of alteration (in %) was calculated by the ratio between the maximal signal alteration value (X) and the baseline value (Y). The signal alteration time window (Z) was measured until the signal recovered ninety percent (90%) of its initial value (Fig. 4).

3.2. Matrix effects

Table 3 presents the results (480 values) obtained for the eight substances as a function of the sample pre-treatment with

Table 2
Switching times for every extraction support

	LiChrospher RP-4 ADS (0.8 mL min^{-1})	LiChrospher RP-4 ADS (4 mL min^{-1})	Oasis HLB (4 mL min^{-1})	Cyclone Turboflow HTLC (4 mL min^{-1})	Chromolith Flash (4 mL min^{-1})
Loading step (min)	0–4	0–1	0–1	0–1	0–1
Transfer step (min)	4–8	1–3	1–3	1–3	1–3
Reconditioning step (min)	8–10	3–10	3–10	3–10	3–10

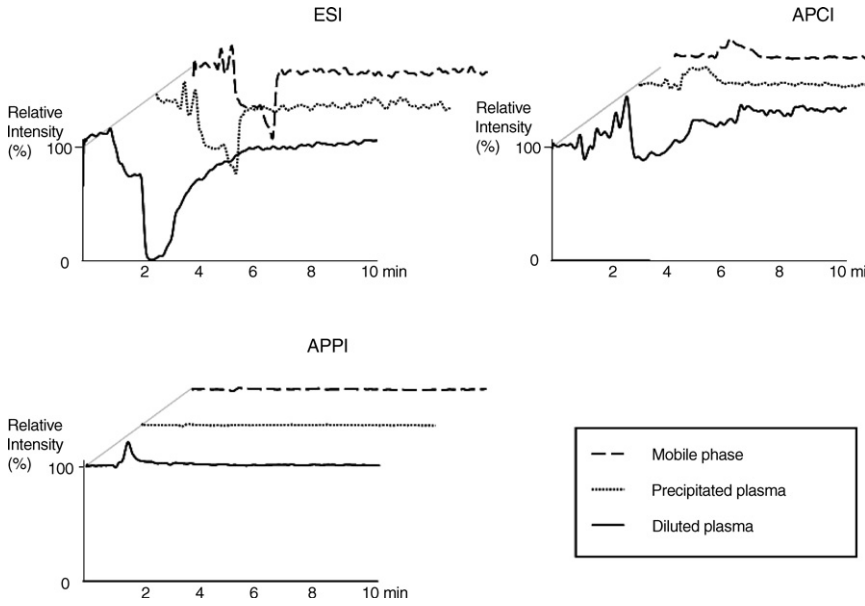

Fig. 3. TIC profiles of sample injections on the Chromolith Flash support.

the three API sources. It is important to note that diluted plasma directly injected in the extraction support led to an important signal alteration at the beginning of the analysis. This is especially pronounced with the ESI source, where the signal alteration time window was about 4.5 min for the Chromolith Flash and the LiChrospher RP-4 ADS used at the conventional flow rate (0.8 mL min^{-1}), while for all other supports it was about 2 min. This larger alteration time window was attributed to the geometry of the cartridge for the Chromolith Flash (25 mm × 4.6 mm i.d.) and the low flow rate applied with the LiChrospher RP-4 ADS. Matrix effect time windows with the Cyclone HTLC support were between 2 and 3 min in ESI and about 6 min in APPI but lasted much longer in APCI, without any increase in back-pressure.

No back-pressure increase was noticed with the LiChrospher RP-4 ADS extraction support at 0.8 or 4 mL min^{-1} with injections of precipitated or diluted plasma. Thus, the LiChrospher RP-4 ADS support can accept the direct injection of plasma at a low flow rate with less residual endogenous substances than other supports. In comparison with previous results [32], dimensions of the Oasis HLB cartridge were different, since a 20 mm × 2.1 mm i.d. was used instead of a 50 mm × 1 mm i.d. Thus, the applied loading flow rate (4 mL min^{-1}) was probably not sufficient to assure eddy strengths and the complete removal of large molecules as confirmed by the column pressure increase between each analysis (about 1–2 bar per injection). As already recommended with the Cyclone extraction support, the new design of the Oasis HLB cartridges requires a preliminary protein precipitation or the application of a very large flow rate (ca. 16 mL min^{-1}), which cannot be achieved with conventional HPLC instrumentation. Since the signal alteration was due to the presence of interfering compounds (e.g. proteins) retained by the extraction support during the loading step, protein precipitation reduced drastically or suppressed the matrix effects for all tested compounds and API sources, as presented in Table 3.

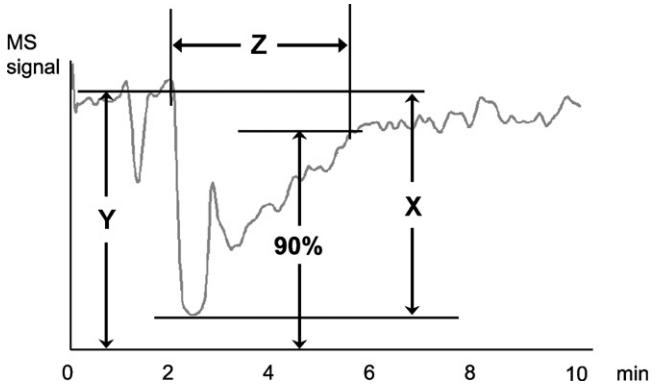

Fig. 4. Maximal signal alteration value (*X*), baseline value (*Y*) and matrix effect time window (*Z*).

For a given sample pre-treatment (dilution or precipitation) and as a function of the extraction support, a different signal alteration was recorded according to the API source. APCI and APPI produced signal enhancements while ESI showed signal suppression when endogenous compounds were eluted. This opposite behavior could be due to the ionization process taking place in the gaseous phase in APCI and APPI whereas in ESI this occurs in the liquid phase. It can be noted that Wang et al. [36] and Hsieh et al. [39,40] observed signal suppression with APCI and APPI sources but data were acquired with different analytes and apparatus. With ESI, matrix effects were totally removed (28 cases out of 40) or strongly reduced after protein precipitation. With APCI, matrix effects were also totally removed or drastically diminished (33 of 40) after PP while seven cases presented an important remaining signal alteration. Finally, with APPI, protein precipitation allowed the total removal of matrix effects in all cases. With precipitated plasma, it therefore appeared that APPI was the least sensitive source to system and matrix effects while ESI was the most sensitive in this regard. Sensitivity toward matrix effects was analyte dependent and the most affected signal was attributed to VD3, while

Table 3
Results of matrix effect (*X*/*Y*) in percent and time window (*Z*) in minutes on each XIC with each source and each extraction support (LiChrospher RP-4 ADS used at 0.8 mL min^{-1} (ADSs), LiChrospher RP-4 ADS used at 4.0 mL min^{-1} (ADSf), Chromolith Flash (Flash), Cyclone Turboflow HTLC (Turbo) and Oasis HLB (HLB))

		Diluted plasma					Precipitated plasma				
		ADSs	ADSf	Flash	Turbo	HLB	ADSs	ADSf	Flash	Turbo	HLB
TIC											
ESI	ME	−61%	−100%	−68%	−100%	−100%	0%	0%	0%	0%	0%
	Time window	4.6	2.2	4.9	2.7	3.2	0.0	0.0	0.0	0.0	0.0
APCI	ME	48%	71%	120%	120%	114%	0%	0%	0%	3%	−60%
	Time window	4.0	4.3	5.7	6.4	3.0	0.0	0.0	0.0	0.3	0.3
APPI	ME	21%	70%	154%	107%	146%	0%	0%	0%	0%	0%
	Time window	3.6	1.5	5.2	1.6	2.1	0.0	0.0	0.0	0.0	0.0
MTD											
ESI	ME	−64%	−100%	−58%	−100%	−100%	0%	0%	0%	0%	0%
	Time window	4.4	2.3	4.5	2.7	3.1	0.0	0.0	0.0	0.0	0.0
APCI	ME	56%	74%	95%	107%	72%	0%	0%	0%	49%	−79%
	Time window	3.9	1.9	5.6	5.8	3.0	0.0	0.0	0.0	0.4	0.3
APPI	ME	19%	68%	170%	86%	134%	0%	0%	0%	0%	0%
	Time window	2.0	1.5	5.5	1.9	2.9	0.0	0.0	0.0	0.0	0.0
EDDP											
ESI	ME	−64%	−100%	−60%	−100%	−100%	0%	0%	0%	0%	0%
	Time window	4.3	2.6	5.2	2.8	3.2	0.0	0.0	0.0	0.0	0.0
APCI	ME	35%	120%	153%	241%	155%	−23%	−36%	0%	27%	−65%
	Time window	3.9	3.4	5.7	6.0	2.9	4.1	0.5	0.0	0.6	0.3
APPI	ME	0%	136%	391%	161%	338%	0%	0%	0%	0%	0%
	Time window	0.0	1.4	4.6	1.8	2.3	0.0	0.0	0.0	0.0	0.0
FLX											
ESI	ME	−64%	−100%	−58%	−100%	−100%	0%	0%	0%	0%	0%
	Time window	4.4	2.3	4.5	2.7	3.1	0.0	0.0	0.0	0.0	0.0
APCI	ME	56%	74%	95%	107%	72%	0%	0%	0%	49%	−79%
	Time window	3.9	1.9	5.6	5.8	3.0	0.0	0.0	0.0	0.4	0.3
APPI	ME	19%	68%	170%	86%	134%	0%	0%	0%	0%	0%
	Time window	2.0	1.5	5.5	1.9	2.9	0.0	0.0	0.0	0.0	0.0
NFLX											
ESI	ME	−64%	−100%	−73%	−100%	−100%	0%	0%	0%	0%	0%
	Time window	4.2	2.0	4.5	3.5	2.7	0.0	0.0	0.0	0.0	0.0
APCI	ME	59%	120%	392%	380%	343%	0%	0%	0%	33%	−56%
	Time window	6.1	4.1	5.7	6.7	3.0	0.0	0.0	0.0	0.3	0.3
APPI	ME	29%	281%	754%	327%	643%	0%	0%	0%	0%	0%
	Time window	1.3	1.7	4.1	2.2	2.5	0.0	0.0	0.0	0.0	0.0
FLZ											
ESI	ME	−61%	−100%	−93%	−100%	−100%	−15%	−22%	0%	15%	17%
	Time window	4.2	1.7	4.2	1.9	2.3	1.1	1.0	0.0	0.5	0.4
APCI	ME	43%	54%	75%	100%	−73%	0%	0%	0%	29%	−53%
	Time window	4.0	1.4	5.7	1.0	3.0	0.0	0.0	0.0	0.3	0.3
APPI	ME	0%	22%	56%	48%	36%	0%	0%	0%	0%	0%
	Time window	2.1	0.5	5.3	0.9	2.8	0.0	0.0	0.0	0.0	0.0
NFLZ											
ESI	ME	−60%	−100%	−85%	−100%	−100%	−10%	−14%	−12%	0%	0%
	Time window	4.2	1.4	4.5	2.1	2.8	1.1	0.9	1.8	0.0	0.0
APCI	ME	77%	98%	611%	248%	342%	0%	0%	46%	65%	−100%
	Time window	6.1	3.9	3.9	6.5	3.0	0.0	0.0	1.0	0.5	0.3

Table 3 (*Continued*)

		Diluted plasma					Precipitated plasma				
		ADSs	ADSf	Flash	Turbo	HLB	ADSs	ADSf	Flash	Turbo	HLB
APPI	ME	0%	67%	196%	83%	128%	0%	0%	0%	0%	0%
	Time window	0.0	1.5	5.1	1.8	3.0	0.0	0.0	0.0	0.0	0.0
VD3											
ESI	ME	−85%	−95%	−52%	−89%	−79%	25%	23%	0%	0%	−12%
	Time window	4.0	2.1	3.3	2.0	5.0	4.0	2.2	0.0	0.0	1.0
APCI	ME	134%	484%	1833%	1474%	755%	0%	0%	0%	−100%	0%
	Time window	5.8	5.8	2.9	5.6	5.7	0.0	0.0	0.0	0.4	0.3
APPI	ME	64%	292%	1118%	850%	1349%	0%	0%	0%	0%	0%
	Time window	4.4	1.4	2.8	1.2	1.1	0.0	0.0	0.0	0.0	0.0
MTX											
ESI	ME	−65%	−100%	−100%	−100%	−100%	−37%	−29%	0%	0%	0%
	Time window	5.1	2.6	4.6	2.1	2.8	4.2	1.2	0.0	0.0	0.0
APCI	ME	46%	46%	68%	81%	75%	0%	0%	0%	0%	0%
	Time window	4.1	9.1	5.7	8.9	3.0	0.0	0.0	0.0	0.0	0.0
APPI	ME	0%	24%	57%	20%	44%	0%	0%	0%	0%	0%
	Time window	0.0	1.4	4.2	1.1	1.2	0.0	0.0	0.0	0.0	0.0

EDDP and NFLX presented an intermediate behavior according to the extraction support and source combination. Moreover, MTX and FLZ exhibited the lowest sensitivity towards matrix effects in APCI and APPI regardless of which extraction support was used.

Fig. 5 presents the concentration measured on the basis of a signal to noise ratio (S/N) of 10. Except for VD3, ESI was the most sensitive ionization source for the selected analytes with a detected concentration of about 1 ng/mL. APCI was about four times less sensitive, followed by APPI (ca. 10 times less sensitive). Therefore, the choice of the ionization source must be made considering two following criteria: matrix effect influence and detection sensitivity.

3.3. Multivariate analysis

For a simplified data representation, principal component analysis (PCA) was chosen to summarize the information obtained due to the numerous variables (i.e. relative signal intensity alteration and time window) in a simple graphical display with minimal loss of information and to assess relationships between variables and individuals (extraction support with and without precipitated plasma). Because part of the information expressed in higher latent variables remained inaccessible, hierarchical cluster analysis (HCA) based on the application of Ward linkage rules and Euclidian distance calculations was used. HCA was performed on the principal coordinates corresponding to 95% of the total variance to obtain tree diagrams (dendograms). The latter were reported on the PCA representation to confirm the identification of groups of extraction supports and to combine the bi-dimensional graphical visualization with the multi-dimensional clustering afforded by HCA (Fig. 6).

All variables were well represented in the first two PC axes where important correlations were observed. For the sake of clarity, an average vector was used to indicate the signal alteration and time window, respectively. As presented in Fig. 6,

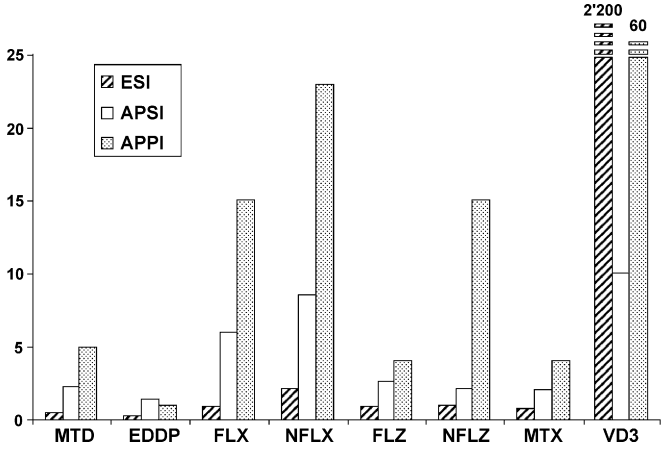

Fig. 5. Analytes sensitivity (S/N = 10) with the tested ionization sources.

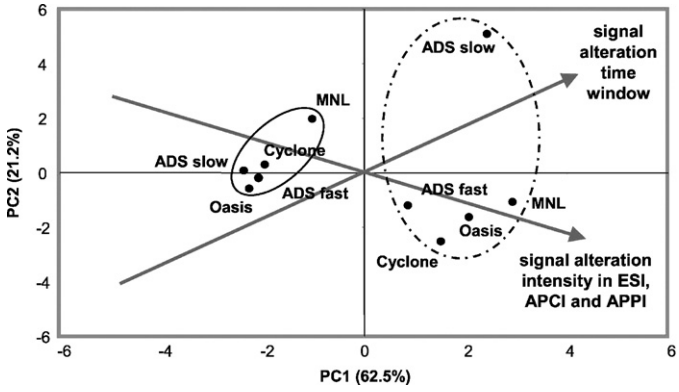

Fig. 6. Global principal components analysis. Dot line: diluted plasma. Continuous line: precipitated plasma.

PCA demonstrated a good clusterization of the supports with about 80% of the total information explained by the first two axes. Two groups were clearly distinguished according to the sample pre-treatment. The first group of supports contained extraction supports which received precipitated plasma while the second group received diluted plasma. Because both signal alterations (intensity and duration) increased from left to right, it can be observed that protein precipitation drastically decreased the observed matrix effect for all extraction supports. Furthermore, with diluted plasma, extraction support behaviors were more dispersed. As presented in Fig. 6, LiChrospher RP-4 ADS used at 0.8 mL min^{-1} (ADS slow) exhibited a significantly different behavior from other supports (lower but longer matrix effects) certainly due to the low applied flow rate. After protein precipitation, this support was clusterized with large particle supports and LiChrospher RP-4 ADS loaded at a high flow rate. Therefore, it can be concluded that PP is very efficient in terms of decreasing matrix effects when used in combination with an on-line extraction procedure.

These results show that the choice of the extraction support is not critical when a PP procedure is achieved prior to the injection in the column-switching system. Because protein precipitation with ACN combined with on-line extraction procedures provides cleaner samples, it will enhance the analytical column lifetime due to the removal of more than 92% of the proteins present in human plasma [50]. Furthermore, in order to avoid quantification problems in the case of real analysis, it is of utmost importance to perform an appropriate chromatographic separation allowing the analytes to be removed from the matrix effect window. When not possible, the use of deuterated internal standard (I.S.) remains of primary importance to get rid of signal modification problems.

4. Conclusion

Matrix effects were compared with ESI, APCI and APPI ionization sources with eight compounds in human plasma and with on-line extraction procedures. Two types of samples were tested: diluted human plasma and precipitated human plasma with ACN. Extracted ion current (XIC) was used for data analysis. The effects of the system were removed from the data to obtain only the matrix effect and the latter was then characterized *via* its signal alteration intensity and time window as a function of the source and extraction support. On the basis of the large number of results obtained, PCA was performed. It emerged that direct injection of plasma after simple dilution generated matrix effects and the selected supports exhibited different behaviors. Nevertheless, a PP step before sample injection provided clean samples and minimized the differences among the behaviors of the supports. Therefore, the choice of the support is not of major importance once a PP step is performed before sample injection. With a protein precipitation procedure prior to on-line SPE–LC–API/MS, APPI was the least affected by matrix effects, followed by APCI and then by ESI. Finally, taking the analyte out of the matrix effect time window with a good chromatography is strongly recommended.

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