Influence de la préparation d'échantillons biologiques en LC-API-MS

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THESE

présentée à la Faculté des sciences de l'Université de Genève
pour obtenir le grade de Docteur ès sciences, mention chimie
par

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de
Genève (GE)

Thèse N° 4047

GENEVE
Atelier ReproMail
2008

UNIVERSITÉ
DE GENÈVE

FACULTÉ DES SCIENCES

Doctorat ès sciences
mention chimie

Thèse de *Monsieur Ivano Cédric MARCHI*

intitulée :

"Influence de la préparation d'échantillons biologiques en LC-API-MS"

La Faculté des sciences, sur le préavis de Messieurs J.-L. VEUTHEY, professeur ordinaire et directeur de thèse (Section des sciences pharmaceutiques, Laboratoire de chimie analytique pharmaceutique), S. RUDAZ, docteur et codirecteur de thèse (Section des sciences pharmaceutiques, Laboratoire de chimie analytique pharmaceutique), M. BORKOVEC, professeur ordinaire (Département de biochimie), Ph. CLEON, docteur (SANOFI, Laboratoire d'analyse, Centre de développement des procédés, Neuville sur Saône, France), D. ORTELLI, docteur (Département de l'économie et de la santé, Service de la consommation et des affaires vétérinaires, Genève, Suisse) et de Madame M.-C. HENNION, professeur (Laboratoire Environnement et chimie analytique, Ecole supérieure de physique et chimie industrielles, Paris, France), autorise l'impression de la présente thèse, sans exprimer d'opinion sur les propositions qui y sont énoncées.

Genève, le 18 décembre 2008

Thèse - 4047 -

Le Doyen, Jean-Marc TRISCONE

N.B.- La thèse doit porter la déclaration précédente et remplir les conditions énumérées dans les "Informations relatives aux thèses de doctorat à l'Université de Genève".

Nombre d'exemplaires à livrer par colis séparé à la Faculté : - 4 -

“What was possible for me is possible for you.”

Frederick Douglass

A Doudou d’Yêu

A ma famille

Remerciements

Les travaux présentés dans cette thèse ont été réalisés entre janvier 2004 et décembre 2008 au Laboratoire de Chimie Analytique Pharmaceutique (LCAP) de l'Université de Genève, sous la direction du Professeur Jean-Luc Veuthey et du Docteur Serge Rudaz.

Je tiens à remercier chaleureusement le Professeur Jean-Luc Veuthey, mon directeur de thèse, pour m'avoir accueilli dans son laboratoire, pour l'intérêt qu'il a porté à ces travaux, pour la confiance qu'il m'a témoignée tout au long de cette thèse ainsi que pour ses judicieux conseils.

Je remercie également le Docteur Serge Rudaz pour sa précieuse contribution au traitement de données ainsi que pour les nombreux échanges d'idées qui ont jalonné tout ce parcours de thèse.

Un grand merci aussi à tous mes collègues passés et actuels pour leur soutien, leur aide, leur collaboration à ce travail ainsi que pour l'excellente ambiance qu'ils font régner dans le laboratoire.

Je souhaite finalement remercier de tout cœur Dao, ma famille et mes amis pour leur soutien et leurs encouragements.

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Avant propos

Le Laboratoire de Chimie Analytique Pharmaceutique (LCAP, Université de Genève) est spécialisé dans l'analyse de composés pharmaceutiques dans les matrices biologiques par chromatographie liquide (LC) et électrophorèse capillaire (CE). Au laboratoire, la LC est utilisée en couplage avec divers modes de détection tels l'ultra-violet (UV) ou le détecteur à barrettes de diodes (DAD), le détecteur évaporatif à diffusion de la lumière (ELSD) ou encore la spectrométrie de masse (MS). Cette dernière est actuellement considérée comme étant la détection de choix pour l'analyse des matrices biologiques. Cependant, la réponse obtenue pour les analytes peut être altérée par la co-élution de composés présents dans les matrices biologiques (effet matrice). L'analyse de composés pharmaceutiques dans de telles matrices requiert donc une étape de préparation d'échantillon de manière à éliminer, partiellement du moins, ces composés interférents et par-là même limiter les effets matrice. Cette étape est souvent considérée comme le facteur limitant du processus analytique en terme de temps. Des méthodes de préparation d'échantillon rapides sont donc apparues, telles l'extraction en phase solide (SPE) sur des plaques multi-puits (i.e. 96, 384) ou encore l'utilisation de supports d'extraction couplés en ligne avec la LC. Parmi ces supports, les phases à accès restreint (RAM) ont été largement étudiées par le Docteur Didier Ortelli dans sa thèse intitulée "*Utilisation de la chromatographie liquide couplée à la spectrométrie de masse pour l'analyse de médicaments dans les fluides biologiques*" (Université de Genève, Thèse n° 3296, 2001). Les deux autres types de supports utilisés en ligne et commercialement disponibles sont les phases à larges particules (LPS) et les phases monolithiques. Ils ont fait l'objet d'une partie des travaux de thèse du Docteur Sandrine Souverain, intitulée "*Extraction en ligne sur support solide pour l'analyse de composés pharmaceutiques contenus dans des matrices biologiques par chromatographie liquide - spectrométrie de masse*" (Université de Genève, Thèse n° 3520, 2004). Dans ses travaux, le Docteur Souverain s'est également attaché à évaluer des autres types de traitement d'échantillon, à savoir l'extraction liquide-liquide (LLE) et la précipitation de protéines (PP) particulièrement adaptée aux matrices sanguines (sang complet, plasma, sérum). Finalement, les avantages et inconvénients de l'utilisation de deux sources d'ionisation à pression atmosphérique commercialement disponibles dédiées au couplage LC-MS, à savoir l'électrospray (ESI) et l'ionisation chimique à pression atmosphérique (APCI), ont été étudiés pour l'analyse de composés modèles dans des matrices sanguines.

Les travaux faisant l'objet du présent manuscrit s'inscrivent dans la continuité des deux thèses précitées. L'objectif est d'investiguer les domaines encore inexplorés de la préparation d'échantillon par SPE et d'étudier l'utilisation de la photo-ionisation à pression atmosphérique (APPI) pour le couplage de la LC à la MS dans le cadre de l'analyse de composés pharmaceutiques dans les matrices biologiques.

Structure de la thèse

Le présent manuscrit se divise en trois chapitres. Le **chapitre 1** décrit, après une courte introduction théorique, le travail de thèse *via* le résumé en français des articles publiés, en cours de publication ou soumis à publication constituant le corps du **chapitre 2**. Pour terminer, le **chapitre 3** comprend les conclusions et perspectives.

Les principaux thèmes abordés dans cette thèse ainsi que les articles illustrant ce travail sont reportés ci-après :

Utilisation de la SPE en plaques multi-puits et en ligne pour la préparation d'échantillon dans le cadre de l'analyse de composés pharmaceutiques dans les fluides biologiques

Dans ce travail de thèse, divers aspects de la SPE, n'ayant pas encore été abordés dans le laboratoire, ont été investigués. La SPE a tout d'abord été évaluée dans sa configuration en ligne en mode commutation de colonnes. Le prétraitement d'échantillon par précipitation de protéines (PP) a été comparé, en terme d'effets matrice générés, à une simple dilution (*dilute and shoot*) sur les trois types de supports commercialement disponibles, à savoir les RAM, les LPS et les monolithes (**article II**). Le développement de méthode en SPE pour l'analyse multi-analytes a ensuite été investigué, notamment dans le cadre de la sélection de composés représentatifs pour l'optimisation des conditions d'extraction (**article III**). Finalement, une classification systématique et exhaustive des effets matrice potentiellement rencontrés lors de l'analyse de matrices biologiques par SPE et LC-MS a été proposée (**article IV**).

L'APPI pour le couplage de la LC à la MS

Le second objectif de ce travail de thèse a été d'apprécier les apports et inconvénients de l'utilisation de la photo-ionisation à pression atmosphérique (APPI) dans le domaine de l'analyse de composés pharmaceutiques dans les fluides biologiques. Dans cette optique, une recherche bibliographique ayant fait l'objet d'une publication (revue) a été menée (**article I**). Ensuite, la susceptibilité de l'APPI aux effets matrice suite à la préparation d'échantillons plasmatiques sur divers supports SPE en ligne (**article II**) a été évaluée et comparée à l'ESI et l'APCI. Pour terminer, l'aspect quantitatif a été étudié *via* le développement et la validation d'une méthode pour l'analyse de l'aprazolam, du flunitrazépam et de leurs principaux métabolites dans le sang total par SPE et LC-APPI-MS (**article V**).

Communications scientifiques

Ce travail de thèse a fait l'objet d'articles publiés dans des revues scientifiques à comité de lecture ainsi que de présentations sous forme de communications orales et de posters, ceci dans le cadre de congrès nationaux et internationaux. A l'issue du congrès SBCA 2005, le poster intitulé '' *Traitement et analyse d'échantillons plasmatiques en chromatographie liquide – spectrométrie de masse : étude des effets de suppression de signal* '' a reçu le second prix du Jury. Au moment de l'impression de cette thèse, 2 articles ont été publiés, 3 sont sous presse et 1 est en cours de rédaction et devrait paraître courant 2009.

Publications

I. **Atmospheric pressure photoionization for coupling liquid-chromatography to mass spectrometry : a review.**
I. Marchi, S. Rudaz, J.-L. Veuthey, *Talanta* (*In press*, doi:10.1016/j.talanta.2008.11.031)

II. **Evaluation of the influence of protein precipitation prior to on-line SPE-LC-API/MS procedures using multivariate data analysis**
I. Marchi, S. Rudaz, M. Selman, J.-L. Veuthey, *Journal of Chromatography B*, 845 (2007), 244-252.

III. **Sample preparation development and matrix effects evaluation for multianalyte determination in urine**
I. Marchi, S. Rudaz, J.-L. Veuthey, *Journal of Pharmaceutical and Biomedical Analysis* (*In press*, doi:10.1016/j.jpba.2008.11.040)

IV. **Characterization and classification of matrix effects in biological samples analysis**
I. Marchi, S. Rudaz, J.-L. Veuthey, (en rédaction)

V. **Development and validation of a liquid chromatography-atmospheric pressure photoionization-mass spectrometry method for the quantification of alprazolam, flunitrazepam, and their main metabolites in haemolysed blood**
I. Marchi, J. Schappler, J.-L. Veuthey, S. Rudaz, *Journal of Chromatography B* (*In press*, doi:10.1016/j.jchromb.2008.12.002)

VI. **Fast Analysis of Doping Agents by Ultra Performance LC (ANNEXE)**
I. Marchi, D. Nguyen, D. Guillarme, L. Mateus-Avois, M. Saugy, S. Rudaz, J.L. Veuthey, *G. I. T. Laboratory Journal, Europe*, 11 (2007) 14-15.

Communications orales

- **Chemometric tools to simplify method development : screening of doping agents in urinary samples**

  I. Marchi, S. Rudaz, J.-L. Veuthey

  Fall Meeting 2007 de la Société Suisse de Chimie, Septembre 2007, Lausanne (Suisse).

- **Evaluation of matrix effects after on-line extraction procedures with commercially available ESI, APCI and APPI sources**

  I. Marchi, M. Selman, S. Rudaz, J.-L. Veuthey

  10th International Symposium on Biochromatography (SBCN 2006), Avril 2006, Lille (France).

- **Sample preparation procedures prior to LC-MS and CE-MS**

  I. Marchi, J. Schappler, D. Guillarme, S. Rudaz, J.-L. Veuthey; *Drug Analysis 2006*, Mai 2006, Namur (Belgique).

- **Traitement des fluides biologiques avant l'analyse par LC-MS et CE-MS**

  I. Marchi, J. Schappler, D. Guillarme, S. Rudaz, J.-L. Veuthey; *Forum Labo 2006*, Mars 2006, Paris (France).

Posters

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

  I. Marchi, S. Rudaz, J.-L. Veuthey

  Fall Meeting 2006 de la Société Suisse de Chimie, Octobre 2006, Zürich (Suisse). Poster numéro 39.

- **Traitement et analyse d'échantillons plasmatiques en chromatographie liquide – spectrométrie de masse : étude des effets de suppression de signal**

  I. Marchi, M. Selman, S. Rudaz, J.-L. Veuthey

  1er Symposium de Biologie et Chime Analytiques- de la molécule au protéome (SCBA 2005), Septembre 2005, Montpellier (France). Poster numéro P-233.

  2ème PRIX DU JURY

Abréviations

Les abréviations utilisées dans ce manuscrit sont répertoriées ci-dessous. Elles sont pour la grande majorité reconnues par la communauté scientifique.

acronyme	signification
AA	anion attachment
ACN	acetonitrile
ACT	acetone
APCI	atmospheric pressure chemical ionization
API	atmospheric pressure ionization
APPI	atmospheric pressure photoionization
BGE	background electrolyte
CAL	calibrator
CE	capillary electrophoresis
CZE	capillary zone electrophoresis
CI	chemical ionization
CV	coefficient de variation
CX	charge exchange
DAD	diode array detection
EA	electronic affinity
EC	electron capture
EDDP	2-ethylidene-1,5-dimethyl-1,3-diphenylpyrrolidine (methadone metabolite)
EI	electron ionization
ESI	electrospray ionization
EtOH	ethanol
FID	flame ionization detector
FLX	fluoxetine
FLZ	flunitrazepam
HCA	hierarchical cluster analysis
HLB	hydrophilic-lipophilic balanced copolymer
HPLC	high performance liquid chromatography
HT-UHPLC	high temperature ultra high pressure liquid chromatography
ICH	international conference on harmonization
IE	ionization energy
IS	internal standard
LC-MS	liquid chromatography – mass spectrometry
LLE	liquid-liquid extraction
LOD	limit of detection
LOQ	limit of quantification
LPS	large particle size
MAX	mixed-mode anion exchange
MCX	mixed-mode cation exchange
MEEKC	microemulsion electrokinetic chromatography
MEKC	micellar electrokinetic chromatography

MeOH	methanol
MIP	molecularly imprinted polymer
MTD	methadone
MTX	metalaxyl
MS	mass spectrometry
MS/MS	tandem mass spectrometry
Mw	molecular weight
m/z	mass on charge ratio
NFLZ	norflunitrazepam
NFLX	norfluoxetine
PA	protonic affinity
PAH	polycyclic aromatic hydrocarbon
PCA	principal component analysis
PI	photoionization
PID	photoionization detector
PP	protein precipitation
QC	quality control
RAM	restricted access material
RSD	relative standard deviation
SDS	sodium dodecyl sulfate
SE	solvation energy
SFSTP	société française des sciences et techniques pharmaceutiques
SLE	supported liquid extraction
SIM	selected ion monitoring
S / N	signal to noise
SPE	solid phase extraction
SST	system suitability test
TIC	total ion current
UHPLC	ultra high pressure liquid chromatography
UV	ultraviolet
VD3	vitamin D3
VUV	vacuum-ultraviolet
WAX	mixed-mode weak anion exchange
WCX	mixed-mode weak cation exchange
XIC	extracted ion current

Chapitre 1 : Résumé du travail de thèse

PREFACE

Le développement d'un médicament est un processus long et coûteux se divisant en une phase de découverte suivie d'une phase de développement. La découverte, ne faisant l'objet d'aucun test humain, comprend l'identification de la cible thérapeutique et des composés chimiques prometteurs, l'optimisation de ces derniers puis les essais précliniques chez l'animal. La phase de développement est, quant à elle, constituée des tests cliniques de toxicité sur l'être humain, relation dose-effet, efficacité et finalement de pharmacovigilance lors de la commercialisation (Tableau 1).

Tableau 1 : de la découverte à la commercialisation du médicament, adapté de [1;2].

1. Découverte 3 - 10 ans				**2. Développement** 5 - 10 ans			
Sélection de la cible thérapeutique	**Identification des composés prometteurs** (criblage des molecules)	**Optimisation des composés prometteurs** (développement d'analogues structuraux)	**Essais précliniques** (Tests *in vitro* et *in vivo* et chez l'animal)	**Essais cliniques Phase I** (Tests toxicité)	**Essais cliniques Phase II** (Relation dose - effet)	**Essais cliniques Phase III** (Tests efficacité)	**Essais cliniques Phase IV** (Commercialisation - pharmacovigilance)
-	5 - 50'000 molécules	100 - 200 molécules	~20 molécules	1 - 5 molécules	1 - 3 molécules	1 - 3 molécules	1 - 3 molécules
Pas d'essais sur l'être humain				100 - 200 volontaires sains	100 - 500 patients	1'000 - 8'000 patients	> 1'000'000 patients

Lors des essais précliniques puis cliniques, les futurs médicaments sont respectivement testés *in vivo* sur des animaux et des êtres humains, requérant l'analyse des principes actifs dans des matrices biologiques (le plus souvent dans le sang ou l'urine).

Dans un second temps, le médicament est commercialisé et utilisé pour le traitement de pathologies. Des patients soumis à des doses identiques peuvent présenter des concentrations sanguines variables du médicament et de ses métabolites. Par conséquent, si la concentration sanguine du médicament se situe en dessous de la fenêtre thérapeutique, le traitement est inefficace et *a contrario*, pour des taux sanguins se situant dans la fenêtre toxique, la santé du patient est mise en danger. C'est pourquoi, dans le cas de la prise de médicaments présentant une fenêtre thérapeutique étroite et/ou une fenêtre toxique large, le contrôle des taux sanguin ou urinaire est nécessaire afin d'ajuster la posologie, c'est le therapeutic drug monitoring (TDM).
De plus, dans le cas d'abus de médicaments (toxicologie) ou dans le sport (dopage), des analyses de composés pharmaceutiques dans des matrices biologiques sont également requises pour l'identification et/ou la quantification de composés prohibés par l'agence mondiale anti-dopage (www.wada-ama.org).

Néanmoins, l'analyse de composés pharmaceutiques dans les matrices biologiques est un exercice délicat étant donné la très faible proportion d'analytes présents dans l'échantillon comparativement à certains composés endogènes. Dans le domaine de la bioanalyse, la LC-MS s'est donc révélée être une excellente technique d'analyse puisqu'elle présente d'excellentes sélectivité et sensibilité. Toutefois, la présence de composés endogènes peut induire des complications, tant au niveau de la séparation chromatographique que de la détection MS. Par conséquent, l'élimination ou la diminution de la quantité de ces composés dans l'échantillon est un prérequis à l'analyse LC-MS. Pour ce faire, l'opérateur dispose d'un éventail de techniques de préparation d'échantillon dont le choix est guidé par divers critères tels que la sélectivité, le temps, la possibilité d'automatisation ou encore le coût engendré.

Dans la majorité des cas, la préparation d'échantillon est effectuée indépendamment et préalablement à l'analyse. Les méthodes les plus couramment employées sont l'extraction en phase solide (SPE), l'extraction liquide-liquide (LLE) ou encore la précipitation de protéines (PP). Or, depuis l'avènement de techniques séparatives ultra-rapides telles que l'UHPLC et l'HT-UHPLC, les méthodes de préparation d'échantillon sont devenues l'étape limitante du processus analytique en terme de temps. Des méthodes rapides ont donc été développées, généralement par l'automatisation du processus. Dans cette optique sont apparus des robots capables d'effectuer des extractions simultanées de 96 voire 384 échantillons. De plus, la préparation de l'échantillon après son injection dans le système LC-MS est également possible, moyennant son extraction sur un support SPE couplé en ligne à la colonne chromatographique.

La préparation d'échantillon ne permettant généralement que de réduire le contenu en composés interférents, leur présence doit être évaluée en regard de l'interférence possible lors de l'ionisation en MS (effets matrice). Ces derniers peuvent être évalués par deux moyens couramment employés. Le premier consiste en l'infusion post-colonne d'une solution d'analytes générant un signal constant dont l'altération rend compte d'un effet matrice. L'information apportée par l'infusion post-colonne étant purement qualitative, le second moyen permet, lui, de renseigner sur l'aspect quantitatif *via* la comparaison des réponses obtenues pour divers échantillons. Actuellement, trois sources sont commercialisées pour le couplage de la LC à la MS et les effets matrice rencontrés diffèrent pour chacune d'elles. L'electrospray (ESI) est la plus couramment utilisée pour son rendement d'ionisation élevé et sa compatibilité avec les molécules polaires représentant plus de 90% des composés pharmaceutiques. L'ionisation chimique à pression atmosphérique (APCI) et la photoionisation à pression atmosphérique (APPI) sont quant à elles moins employées dans le domaine pharmaceutique, puisqu'*a priori* moins adaptées à l'ionisation de composés polaires. Cependant, il a été constaté que, souvent, les effets matrices rencontrés en APCI et en APPI sont plus faibles que ceux générés en ESI et que les réponses des analytes peuvent être comparables voire supérieures à celles obtenues avec cette dernière.

La première partie de ce chapitre ne s'attachera à décrire que succinctement les méthodes de préparation d'échantillon utilisées au cours de ce travail de thèse, puisqu'une description complète et approfondie a déjà été présentée dans les manuscrits de deux précédentes thèses (voir avant-propos). La seconde partie sera consacrée à la théorie de l'APPI et la troisième partie résumera, quant à elle, les résultats obtenus lors de ce travail de thèse.

1. INTRODUCTION

1.1 Préparation d'échantillons biologiques par SPE

La préparation d'échantillon comprend un vaste ensemble de procédures compte tenu de l'extrême diversité des matrices et des analytes. Les échantillons biologiques principalement utilisés sont l'urine et les matrices sanguines (sang total, plasma et sérum), alors que l'analyse des cheveux/poils, du liquide céphalorachidien (LCR), de l'humeur vitrée, de la salive, de la sueur, des sécrétions gastriques ou encore des fèces est moins courante. Concernant l'extraction de composés pharmaceutiques présents dans des échantillons urinaires et sanguins, les méthodes auxquelles ont communément recours les laboratoires sont 1. la précipitation de protéines (PP) dans le cas des matrices sanguines, 2. l'extraction liquide-liquide (LLE) et 3. l'extraction sur phase solide (SPE). D'autres préparations telles l'ultrafiltration ou la dialyse sont quant à elles moins utilisées. Parmi les trois principales méthodes citées, la PP est souvent utilisée pour sa rapidité de mise en œuvre. En revanche, elle est peu spécifique et son efficacité est relative puisque seule une partie des protéines est précipitée, la proportion dépendant de l'agent précipitant employé [3]. La LLE est plus sélective, permet une élimination efficace des composés endogènes, mène à de bons rendements d'extraction, mais souffre d'une mise en œuvre assez longue. Des cartouches permettant la réalisation d'une LLE assistée sur support solide (SLE) ont récemment été mises sur le marché, proposant ainsi une alternative aux principaux inconvénients de la méthode. Cependant, la littérature ne recense pour le moment que peu d'applications dans ce domaine. Finalement, la SPE est une méthode très sélective puisque de nombreuses phases stationnaires sont commercialisées. De plus, sa mise en œuvre sous diverses formes telles que cartouches, plaques 96 puits et supports d'extraction couplés en ligne à la LC rendent son utilisation adaptable à bon nombre d'applications telles que le suivi thérapeutique (TDM), les études pharmacocinétiques (PK), la toxicologie ou encore la lutte anti-dopage, pour ne citer que les plus courantes.

Le but de cette introduction est de présenter un résumé des aspects théoriques liés à la SPE, puisque ceux-ci ont déjà été largement décrits dans les thèses des Docteurs Sandrine Souverain et Didier Ortelli. Le lecteur intéressé à approfondir les notions présentées ici est invité à consulter leurs manuscrits [4;5].

1.1.1 Principe

L'extraction sur phase solide est une méthode d'extraction apparentée à la LC, puisque les phases stationnaires utilisées en SPE sont du même type que celles utilisées en LC, ne différant parfois que par le diamètre des particules et des pores. Les mêmes mécanismes de rétention sont donc rencontrés.

Il existe des phases stationnaires polaires (phase normale), apolaires (phase inverse) ou de rétention mixte, permettant des rétentions de type hydrophobe et ionique. L'utilisation des premières est cependant peu courante dans le domaine de la bioanalyse, puisque les composés endogènes, en grande majorité polaires, sont retenus sur ce type de supports.

Le déroulement d'une préparation d'échantillon par SPE comprend typiquement trois étapes. L'échantillon est tout d'abord chargé sur le support, les analytes ainsi qu'une partie des interférents étant retenus sur la phase stationnaire. S'ensuit un lavage visant à éluer les composés indésirables et une élution sélective et totale des analytes. Dans le cas d'un mécanisme de *rétention mixte*, deux élutions peuvent être effectuées pour récolter les analytes dans chacune des fractions, les rétentions de type hydrophobe et ionique par exemple pouvant être rompues séparément *via* l'adaptation du solvant et de son pH. Ces supports de rétention mixte sont souvent bien adaptés à l'analyse de composés pharmaceutiques, puisque ces derniers sont, dans une majorité des cas, des bases ou des acides possédant une structure hydrophobe.

1.1.2 Types de phases

1.1.2.1 Phases à base de silice

A l'instar des phases stationnaires des colonnes chromatographiques, la silice est le matériau qui a été le plus utilisé en SPE soit directement, soit *via* un greffage de groupements variés polaires ou apolaires (cyanoalkyle, aminoalkyle ou diols par exemple). Dans le cas de rétention en phase inverse, la silice est greffée avec des groupements hydrophobes tels des chaînes alkyle (C_2, C_4, C_6, C_8, C_{18}) ou des fonctions cyclohexyl (CH), phényl (PH) ou encore cyanopropyl (CNP) (Figure 1) permettant des interactions importantes avec des cinétiques très rapides (Van der Waals, ponts H).

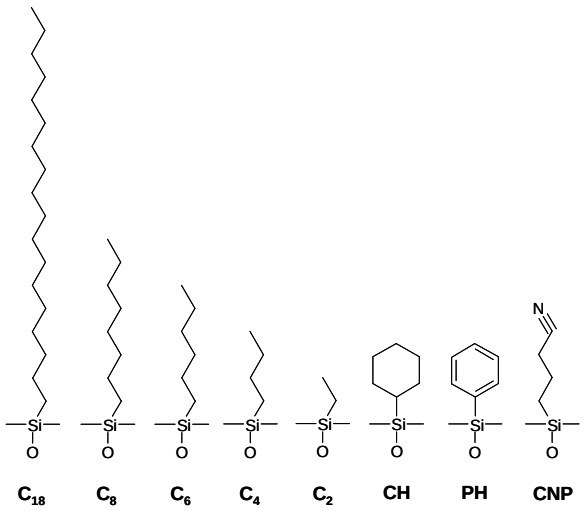
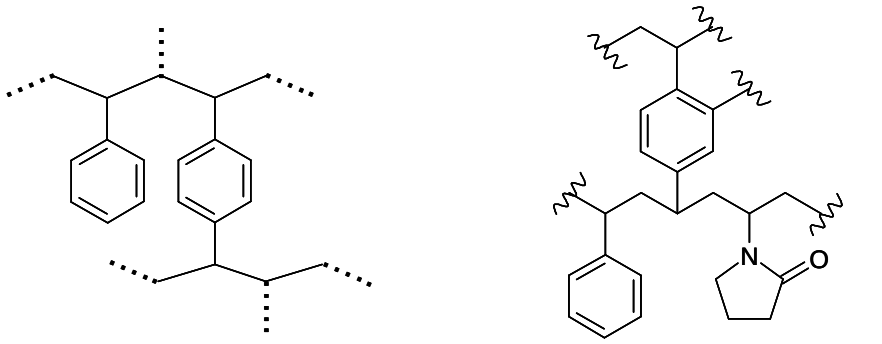


Figure 1 : structure des principaux greffages disponibles sur silice.

Les phases à base de silice sont encore largement utilisées de nos jours mais présentent trois inconvénients majeurs. D'une part, la silice greffée n'est stable qu'à des pH compris entre 2 et 8 et est donc inadaptée à l'extraction de composés nécessitant des phases mobiles d'élution basique. D'autre part, ces phases ne doivent pas être séchées entre chaque étape d'extraction, rendant la manipulation plus délicate. Finalement, dans le cas de silice greffée avec des chaînes alkyle, une étape de déploiement de celles-ci, par percolation de MeOH, est nécessaire pour le conditionnement du support.

1.1.2.2 Phases polymériques

Les phases polymériques sont apparues sur le marché il y a une douzaine d'années et possèdent certains avantages sur la silice. La nature du support polymérique (polystyrène-divinylbenzène, N-vinylpyrrolidone-divinylbenzène, *etc.*) les rend naturellement hydrophobes (Figure 2) et éventuellement polaires, comme dans le second cas où des interactions de type polaire sont directement réalisables *via* le N-vinylpyrrolidone. La modulation de la rétention est également possible par le greffage de groupements polaires ou ioniques, le greffage anionique le plus courant étant le groupement sulfonate (SO_3^-, pKa < 1).



polystyrène-divinylbenzène N-vinylpyrrolidone-divinylbenzène

Figure 2 : structures des polymères polystyrène-divinylbenzène et N-vinylpyrrolidone-divinylbenzène.

L'utilisation des phases polymériques revêt donc plusieurs avantages tels une stabilité étendue au pH (1-12 voire 0-14 suivant les cas), la possibilité de sécher le support entre chaque étape d'extraction, une bonne compatibilité avec les matrices biologiques et l'absence de conditionnement du support avant l'extraction.

Lors de l'analyse d'un composé ou d'une famille de composés de structures proches, la sélectivité de l'extraction peut être accrue, diminuant ainsi les risques de co-extraction de matériel interférent.

L'amélioration de la sélectivité est rendue possible par le greffage d'une molécule jouant le rôle d'anticorps, permettant des liaisons spécifiques de type antigène-anticorps. Ces supports portent le nom d'*immunosorbent*. La réalisation de ce type de greffage est en revanche onéreuse et longue.
Pour pallier ces inconvénients, un autre type de support a été mis au point. Au sein même de la phase stationnaire, des sites de reconnaissance moléculaire permettant une extraction sélective de l'analyte sont créés. Ce type de support est nommé *polymère à empreinte moléculaire* (MIP). Le principe de fabrication est basé sur la polymérisation du matériau (typiquement de l'acide méthacrylique avec un solvant aprotique peu polaire ou apolaire) autour d'une molécule modèle laissant une cavité imprimée dans la structure du support après élimination du composé modèle par lavage. Les avantages de ce type de phase sont une stabilité thermique et chimique améliorées ainsi qu'une fabrication rapide, simple et bon marché. En revanche, l'utilisation de phases à base de méthacrylate nécessite le transfert de l'analyte, souvent par une LLE ou une SPE préalable, dans un solvant compatible avec le support [6].

1.1.3 Automatisation

Outre la qualité de l'échantillon préparé par SPE, le second atout de la SPE est l'automatisation, puisque la préparation d'échantillon en routine est souvent considérée comme l'étape limitante en terme de temps et de variabilité. L'automatisation de la SPE peut se faire *via* l'utilisation de robots gérant l'extraction sur cartouche ou plaque multi-puits. De plus, il existe des supports d'extraction permettant l'extraction de l'échantillon après son injection dans le système analytique (c.f. § 1.1.3.2).

Lorsque l'extraction est effectuée de manière indépendante et préalable à l'injection de l'échantillon, on parle de processus *off-line*. Lorsque l'extraction est automatisée *via* l'utilisation d'un robot pour le transfert automatisé de l'échantillon vers le système chromatographique, le processus est appelé *at-line*, alors que la dénomination *on-line* est réservée à l'utilisation des supports d'extraction décrits au § 1.1.3.2.

1.1.3.1 Plaques multi-puits

Le principe sous-jacent à l'utilisation des plaques multi-puits (48, 96 ou 384) est l'extraction d'un grand nombre d'échantillons dans un court laps de temps, diminuant la durée de préparation par échantillon. Ces plaques correspondent à des cartouches individuelles solidarisées les unes aux autres (Figure 3).

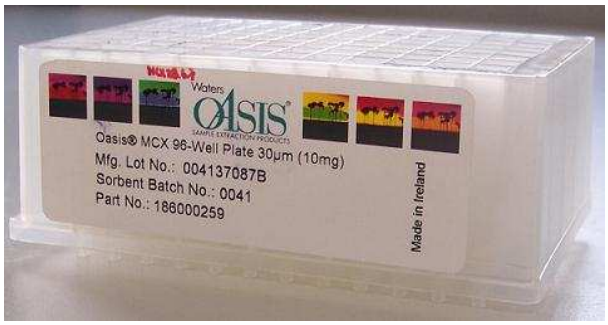


Figure 3 : exemple de plaque 96 puits (Waters).

Le format le plus courant est celui comprenant 96 puits (12 x 8). De paire avec l'utilisation de ce format, des pipettes multi-canaux (8 ou 12) sont disponibles de manière à traiter la plaque par rangées complètes. Des robots sont également commercialisés pour permettre une automatisation totale de l'extraction (dispensation des liquides, application de la pression/dépression, collecte des fractions) sur ce type de format.

1.1.3.2 Supports d'extraction couplés à la chromatographie liquide

L'extraction de l'échantillon après son injection dans le système LC est une alternative rendue possible grâce à l'utilisation de supports d'extraction prévus à cet effet. Ces derniers se présentent sous la forme d'une colonne analytique de dimensions réduites. Comme en SPE classique, l'échantillon est d'abord chargé, les interférents éliminés par lavage et les analytes finalement élués. Trois types de support sont commercialement disponibles, suivant la nature de la phase stationnaire les constituant et permettant l'injection directe de liquides biologiques. Ce paragraphe ne se veut qu'un bref rappel des notions de base, une description détaillée de ce sujet étant disponible dans les thèses des Docteurs Sandrine Souverain et Didier Ortelli.

1.1.3.2.1 Phases à accès restreint

Le plus souvent à base de silice, les RAM permettent l'exclusion des macromolécules des sites d'interaction *via* deux mécanismes, l'un physique et l'autre chimique. La barrière physique est obtenue par des pores de petit diamètre empêchant la diffusion des macromolécules (Figure 4a). L'exclusion chimique est quant à elle rendue possible par le greffage d'une barrière hydrophile constituée de polymères ou de protéines à la surface de la particule (Figure 4b).

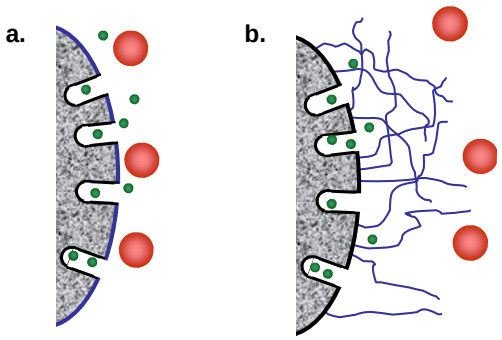


Figure 4 : illustration des barrières (a) physique et (b) chimique [4].

Outre les deux types de barrières rencontrées, la chimie de la surface de la particule peut être différente de celle présente à l'intérieur des pores. Les RAM sont donc classées en quatre familles résumées dans le Tableau 2.

Tableau 2 : classification des familles de RAM.

	barrière physique	barrière chimique
chimie uniforme	Type A	Type C
chimie différente	Type B	Type D

Le diamètre de particules des RAM est généralement de l'ordre de 25 µm.

1.1.3.2.2 Phases à larges particules

Le principe d'exclusion des macromolécules aux sites d'interaction des LPS est directement lié au diamètre élevé des particules. En effet, ces dernières sont du même type que celles utilisées dans les colonnes chromatographiques à polarité de phase inversée (cinétique d'interaction rapide), mais diffèrent de celles-ci par leur taille. Le diamètre de particules des LPS est typiquement compris entre 25 µm et 60 µm, créant des interstices interparticulaires de grand volume (Figure 5).

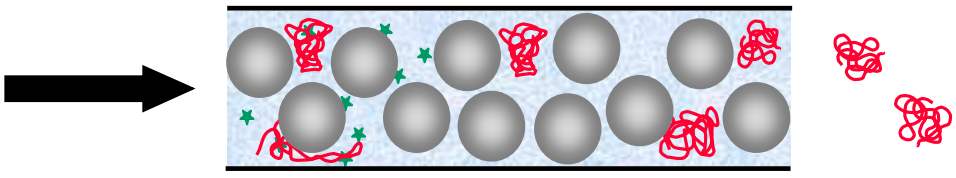


Figure 5 : élution des macromolécules à travers une phase LPS [5].

Ces grosses particules permettent l'application de débits de phase mobile de chargement très importants (de l'ordre de 100 fois supérieur à ceux appliqués sur une colonne analytique de mêmes dimensions externes), puisque la perte de charge est fortement diminuée. La notion de flux turbulent a été avancée pour expliquer le mode d'extraction. Cependant, la limite distinguant un flux laminaire d'un flux turbulent dans un tube rempli n'est pas clairement déterminée.

1.1.3.2.3 Phases monolithiques

Tout d'abord prévus pour des séparations chromatographiques rapides, les caractéristiques des monolithes permettent leur utilisation pour la préparation d'échantillon. Contrairement aux deux types de supports décrits précédemment, ces phases ne sont pas constituées de particules. Ce sont des barreaux d'une seule pièce de grande perméabilité (comparable à celle de colonnes remplies de particules de 11 µm [7]) permettant l'application de hauts débits (5 à 10 fois supérieurs aux débits conventionnels) tout en maintenant une contre-pression acceptable. Ces monolithes possèdent une structure bimodale du réseau poreux composé de macropores (typiquement 2 µm) et mésopores (typiquement 13 nm) dont la taille peut être indépendamment contrôlée lors de la fabrication (Figure 6).

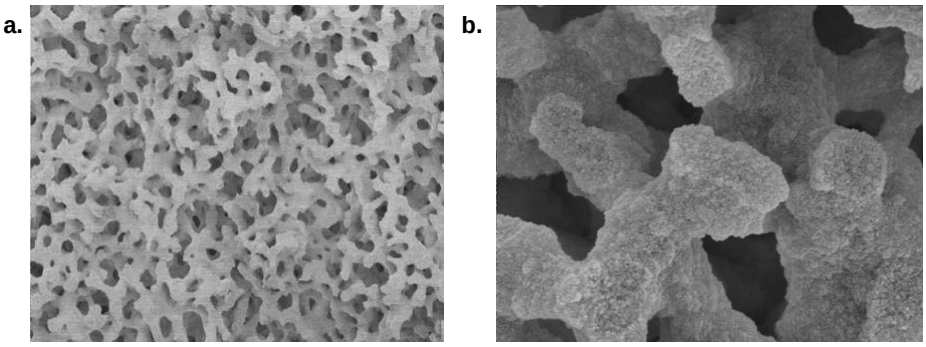


Figure 6 : représentation de la structure (a) macroporeuse et (b) mésoporeuse d'un support monolithique de type Chromolith (Merck).

Les phases stationnaires commercialisées sont de type polymérique ou à base de silice (les plus utilisées dans le domaine pharmaceutique). Ces dernières ont été développées par Tanaka et Nakanishi en 1996 [8] mais présentent une faible résistance aux pH extrêmes. Leur intérêt est également limité puisque le choix du diamètre interne est restreint (4.6 mm, 3.0 mm ou 100 µm) et puisque seuls deux fabricants, Merck et Phenomenex, proposent de telles phases, sous les noms respectifs de Chromolith et Onyx. L'utilisation de support monolithiques pour la préparation d'échantillon présente l'avantage d'une bonne compatibilité avec les matrices biologiques puisque, contrairement aux phases particulaires, ils ne nécessitent pas de frittés aux extrémités de la colonne

pour retenir la phase stationnaire, éliminant ainsi le principal site d'adsorption des composés endogènes.

1.1.3.3 Modes de travail de la SPE

Il existe deux utilisations distinctes de ces supports d'extraction, le mode colonne simple et le mode commutation de colonnes.

1.1.3.3.1 Mode colonne simple

Sur le même et unique support se déroule la préparation de l'échantillon et la séparation des analytes sur le support d'extraction. L'échantillon est tout d'abord chargé, lavé puis l'élution est réalisée avec une composition de phase mobile permettant non pas une élution immédiate des analytes comme en SPE classique, mais permettant une séparation chromatographique des analytes. Ce mode présente l'avantage de ne nécessiter qu'une vanne 6 voies supplémentaire à un appareillage standard, pour diriger les phases de chargement et de lavage vers la poubelle et d'élution vers le détecteur (Figure 7). En revanche, les faibles performances chromatographiques des supports d'extraction ne permettent pas de réaliser des séparations complexes.

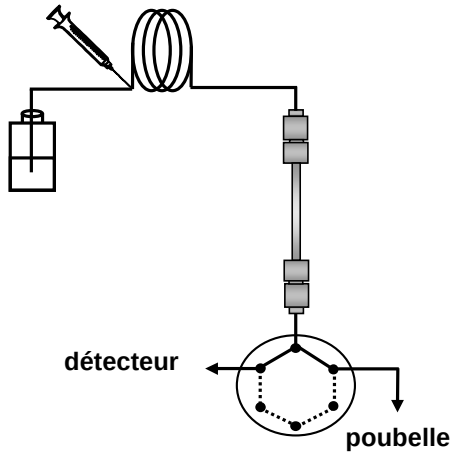


Figure 7 : schéma du montage en mode colonne simple [5].

1.1.3.3.2 Mode commutation de colonnes

L'extraction en ligne par commutation de colonnes, *a contrario* du mode colonne simple, nécessite un support d'extraction pour la préparation de l'échantillon et une colonne chromatographique pour la séparation. La qualité de cette dernière est donc fortement améliorée par rapport au mode colonne simple, mais l'appareillage requiert une pompe binaire supplémentaire pour l'alimentation en phase mobile de la colonne chromatographique (Figure 8).

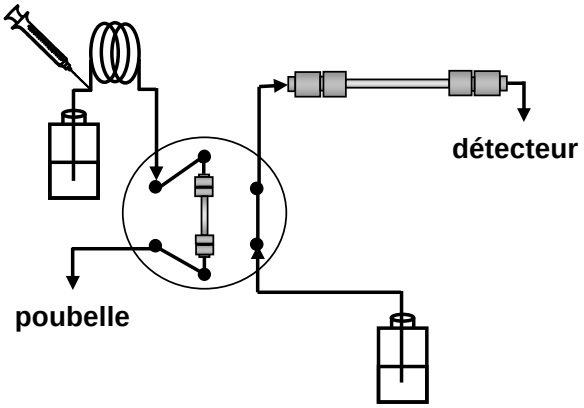


Figure 8 : schéma du montage en mode commutation de colonnes [5].

L'échantillon est d'abord chargé puis le support lavé avant l'élution des composés vers la colonne chromatographique. L'élution étant effectuée avec la phase mobile de séparation, le comportement chromatographique des composés doit être suffisamment différent sur chacun des supports pour permettre une élution immédiate et totale de tous les analytes vers la colonne chromatographique. Durant la séparation, le support d'extraction est quant à lui régénéré avec une phase mobile contenant une importante proportion de solvant organique de manière à éliminer les interférents apolaires retenus lors du chargement de l'échantillon, du lavage puis de l'élution des analytes. La dernière étape consiste à reconditionner le support avec la phase mobile de chargement.

1.2 L'APPI en tant que source d'ionisation pour la LC-MS

Seul un résumé est proposé ici, le lecteur intéressé étant invité à se référer à l'article de revue complet présenté dans le chapitre 2 (article I).

La spectrométrie de masse (MS) est de nos jours considérée comme le détecteur de choix pour le couplage à la chromatographie liquide (LC), compte tenu de sa quasi-universalité, sa sélectivité et sa sensibilité. Le premier couplage de ce type a vu le jour dans les années 1970, mais son utilisation ne s'est répandue que lorsque les sources à pression atmosphérique (API) sont apparues au début des années 90. L'electrospray (ESI) et l'ionisation chimique à pression atmosphérique (APCI) sont les deux sources API les plus couramment utilisées. L'ESI est particulièrement adaptée à l'analyse de composés polaires, le processus d'ionisation se déroulant en phase liquide, alors que l'APCI permet l'ionisation en phase gazeuse de composés moins polaires. La photo-ionisation à pression atmosphérique (APPI) est la dernière-née parmi les sources API et permet l'ionisation de molécule n'étant pas ou mal ionisées en ESI et APCI, comme les composés apolaires [9-11].

La photo-ionisation (PI) a été utilisée à pression réduite dès le milieu des années 1970 en chromatographie gazeuse (GC-PID). Elle permet d'améliorer la sensibilité de composés apolaires (benzène) par rapport au détecteur à ionisation de flamme (FID) alors couramment utilisé et présente une gamme dynamique supérieure à 10^7 [12-15]. De même, les premiers essais de couplage de la photo-ionisation (PI) à la LC voient le jour avec un montage similaire à celui utilisé en GC [16-18].

L'utilisation de l'APPI a été décrite pour la première fois en 1983 par Baim *et al.* dans le couplage avec la spectrométrie à mobilité d'ion (IMS) [19]. Divers exemples d'utilisation de ce type de couplage ont été ensuite proposés [20-25]. C'est en 1991 que le premier couplage de l'APPI avec la MS apparaît [26]. Finalement, l'utilisation de l'APPI pour le couplage de la LC à la MS a été publiée pour la première fois en 2000 par Robb *et al.* [9] et Syage *et al.* [27] (Tableau 3).

Tableau 3 : résumé chronologique de l'utilisation de la PI.

sujet	1ère publication	auteurs
GC-**PID**	années 70	Driscoll *et al.*
LC-**PID**	années 70	Schermund *et al.*
APPI-IMS	1983	Baim *et al.*
APPI-MS	1991	Revel'skii *et al.*
LC-**APPI**-MS	2000	Robb *et al.* & Syage *et al.*

Le nombre de publications faisant appel à la PI a rapidement augmenté depuis son implémentation à pression atmosphérique. En effet, l'utilisation de la PI à pression réduite ne génère que peu d'ions étant donné la faible densité d'échantillon dans la région de l'ionisation [28]. Deux sources APPI sont actuellement commercialisées sous les noms de PhotoSpray et de PhotoMate. La première présente une géométrie axiale basée sur le prototype utilisé par Robb *et al.* en 2000 [9] (Figure 9a) et équipe les appareils Applied Biosystems et Sciex. La seconde, développée par Syage en collaboration avec

Agilent, diffère de sa consoeur par une géométrie orthogonale (Figure 9b) et équipe les MS Agilent et Waters.

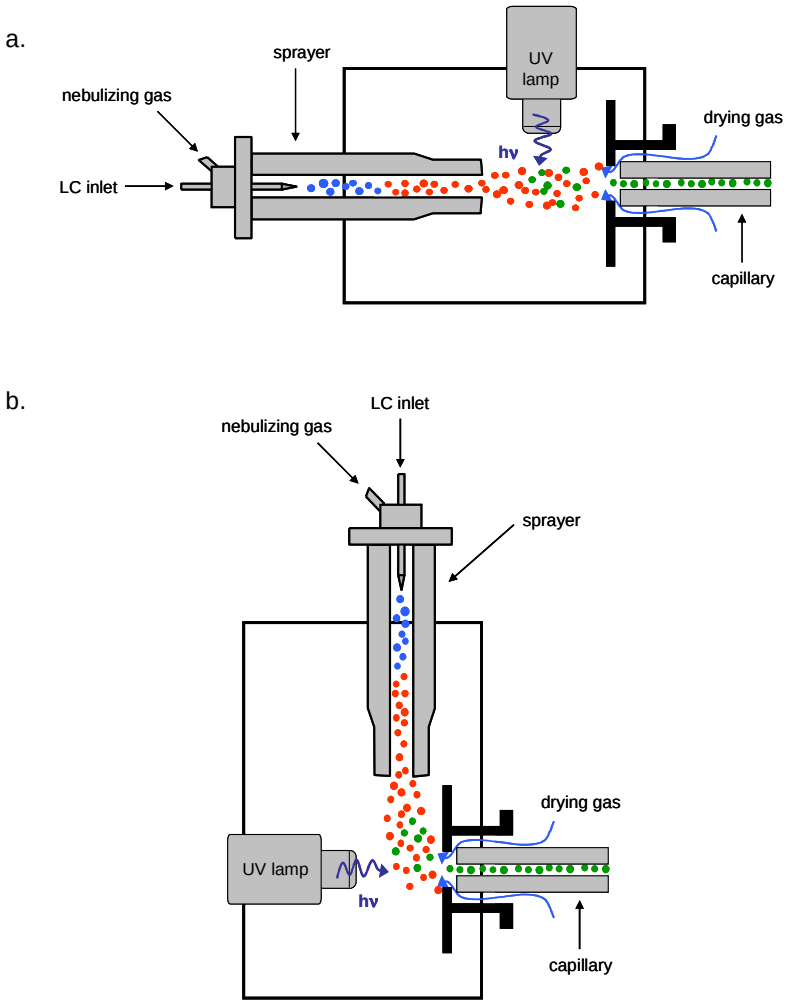


Figure 9 : schémas inspirés d'Agilent des sources a) PhotoSpray et b) PhotoMate.

1.2.1 Théorie

Le principe de l'ionisation en APPI est basé sur l'absorption d'un photon par les composés se trouvant sous forme gazeuse après nébulisation de la phase mobile. L'ionisation se déroule en plusieurs étapes. Tout d'abord, l'absorption d'un photon d'une énergie hν par une molécule fait passer celle-ci à un état excité :

$$M + h\nu \rightarrow M^* \qquad (1)$$

Si l'énergie d'ionisation (IE) de l'analyte M est plus basse que hν, la molécule éjecte un électron menant à la formation d'un radical-cation :

$$M^* \rightarrow M^{+\bullet} + e^- \quad (2)$$

Cependant, dans le cas où l'IE de la molécule est plus élevée que hν, M* est sujet à diverses réactions de désexcitation telles que la photo-dissociation (3), l'émission d'un photon (4) ou encore le *quenching* collisionel avec une molécule non excitée C (5) :

$$M^* \rightarrow A + B \quad (3)$$

$$M^* \rightarrow M + h\nu \quad (4)$$

$$M^* + C \rightarrow M + C^* \quad (5)$$

Dans de tels cas, l'utilisation d'une molécule s'ionisant facilement (dopant D) a été proposée de manière à favoriser l'ionisation de la molécule :

$$D + h\nu \rightarrow D^{+\bullet} + e^- \quad (6)$$

Le dopant sert d'intermédiaire entre le photon et l'analyte. Ses ions présentent une haute énergie de recombinaison et / ou une faible affinité protonique (PA), favorisant un transfert de charge (7) ou de proton (8) vers l'analyte. Le type de transfert dépend de l'affinité électronique (EA) et de la PA de chacune des molécules [9].

$$D^{+\bullet} + M \rightarrow D + M^{+\bullet} \quad \text{si } EA_D > EA_M \quad (7)$$

$$D^{+\bullet} + M \rightarrow [D\text{-}H]^{\bullet} + [M+H]^+ \quad \text{si } PA_M > PA_{[D\text{-}H]\bullet} \quad (8)$$

L'utilisation d'un dopant peut non seulement être prévue pour forcer l'ionisation d'un composé, mais également pour améliorer le rendement d'ionisation d'une molécule dont IE > hν. En effet, étant donné la prédominance de molécules de solvant S, la probabilité de l'ionisation directe de l'analyte est faible. Dans le cas d'une séparation en LC, les molécules de solvant présentes réagissent selon :

$$D^{+\bullet} + nS \rightarrow [D\text{-}H]^{\bullet} + [S_n+H]^+ \quad \text{si } PA_S > PA_{[D\text{-}H]\bullet} \quad (9)$$

$$[S_n+H]^+ + M \rightarrow nS + [M+H]^+ \quad \text{si } PA_M > PA_S \quad (10)$$

La majeure partie des études publiées en APPI recourt à l'utilisation de lampes au krypton, puisque les deux sources commerciales en sont équipées. Ce type de lampe produit des électrons d'une énergie de 10.03eV (λ =123.6nm) et 10.64eV (λ =116.5nm) dans un rapport 4:1 [29;30]. Le krypton est préféré puisque la plupart des analytes (dans le cas de l'analyse de petites molécules) possède une IE plus basse que l'énergie des photons produits alors que les solvants communément utilisés et les gaz présents dans la source (O_2, N_2 *etc.*) présentent, eux, des valeurs supérieures (Tableau 4).

Tableau 4 : IE et PA de divers composés couramment rencontrés en LC-MS

composé	IE (eV)	PA ($kJ \cdot mol^{-1}$)
azote	15.6	493.8 [1]
eau	12.6	691.0 [4]
acétonitrile	12.2	779.2 [3]
oxygène	12.1	421.0 [4]
	Ar : 11.7	
chloroforme	11.4 [3]	n.d.
méthanol	10.8	754.3 [3]
acide acétique	10.7 [4]	783.7 [4]
	Kr : 10.6	
isopropanol	10.2	793.0 [1]
hexane	10.1	n.d.
ammoniac	10.1 [4]	853.6 [4]
	Kr : 10.0	
heptane	9.9	n.d.
isooctane	9.8	n.d.
dimère de methanol	9.7 [2]	899.1 [2]
acétone	9.7	812.0 [3]
tetrahydrofurane	9.4 [1]	822.1 [1]
pyridine	9.2	930.0 [1]
testostérone	9.2 [3]	880.0 [3]
benzène	9.2	750.4 [3]
furane	8.8	803.4 [1]
toluène	8.8	784.0 [3]
	Xe : 8.4	
anisole	8.2 [3]	839.6 [3]
naphtalène	8.1 [3]	802.9 [3]
anthracène	8.1 [3]	877.3 [3]
acridine	7.8 [3]	972.6 [3]
triéthylamine	7.5	981.8 [1]
radical benzyl	7.2 [4]	831.4 [4]

[1] [31]
[2] [32]
[3] [33]
[4] [34]
n.d. : non disponible

Le xenon et l'argon ont aussi été utilisés, mais à moindre échelle. Le xenon produit des photons moins énergétiques mais pénétrant mieux le mélange gazeux. L'argon, *a contrario*, produit des photons plus énergétiques que le krypton (11.7eV), mais ceux-ci sont totalement absorbés par le mélange gazeux après quelques millimètres seulement. Le krypton présente donc le meilleur compromis aux vues des caractéristiques recherchées.

1.2.2 Les solvants en APPI

Ce paragraphe est consacré à l'impact de l'utilisation sur l'APPI des modificateurs organiques les plus couramment utilisés en chromatographie à polarité de phase inversée, à savoir le MeOH et l'ACN.

Globalement, l'utilisation du MeOH permet un meilleur transfert de charge que l'ACN, alors que le transfert de proton n'est que peu ou pas affecté par la nature du solvant utilisé, ceci en absence de dopant (en présence de dopant, voir § 1.2.4.2) [9;30]. Le MeOH joue un rôle d'intermédiaire lors de l'ionisation par transfert de protons. En effet, des dimères de MeOH formés en phase gazeuse [35;36] forment un ion de MeOH protoné par ionisation dissociative :

$$[CH_3OH]_2 + h\nu \rightarrow [CH_3OH]_2^+ + e^- \quad (11)$$

$$[CH_3OH]_2^+ \rightarrow [CH_3OH+H]^+ + CH_3O \text{ (or } CH_2OH) \quad (12)$$

L'ion de MeOH ainsi protoné effectue dans un deuxième temps un transfert de proton vers l'analyte grâce à une PA plus basse que celle de la plupart des analytes [30].

L'ACN, à l'instar du MeOH, participe à l'ionisation par transfert de proton. En dépit de son IE supérieure à celle des photons produits par une lampe au krypton, Marotta *et al.* [28;37] ont montré qu'une molécule d'ACN absorbant un photon s'isomérise en une structure stable (Figure 10) protonée, capable de transfert de proton.

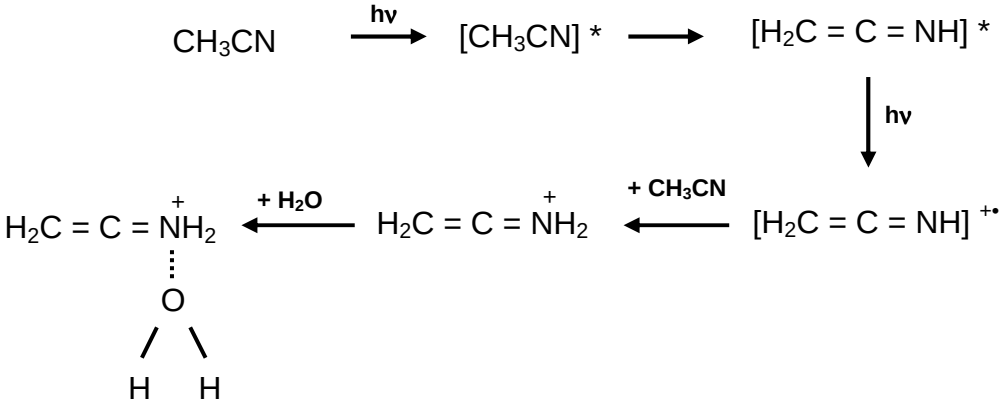


Figure 10 : mécanisme réactionnel de production d'un cluster protoné d'ACN selon [28].

Cependant, selon Short *et al.* [30], deux absorptions consécutives de photon semblent peu probables et l'ionisation d'une molécule d'ACN serait due au transfert d'un hydrogène d'une molécule d'ACN neutre vers une molécule d'ACN excitée, qui suite à divers réarrangements, réalise un transfert de proton vers l'analyte.

1.2.3 APPI assistée par dopant

Le principe de l'ajout d'un composé favorisant l'ionisation n'est pas chose nouvelle. En effet, dès 1994, l'acétone puis le benzène, le toluène et le xylène en 1997 ont été utilisés comme dopants pour améliorer les réponses en PI-IMS. En LC-APPI-MS, l'acétone [38-61] et le toluène [29;51;54;55;62-105] ont été largement étudiés, alors que l'anisole [33;49;51;55;59;60;77;78;106], le benzène [66;107], l'hexafluorobenzène [59;106] et le THF [53] l'ont moins été.

L'ionisation du benzène est directement proportionnelle à sa pression partielle dans la source. A basse pression, les ions $[M]^{+\bullet}$ sont formés et disparaissent à haute pression, formant six ions différents. Parmi ceux-ci, deux sont responsables de l'ionisation ultérieure des analytes. Le radical cation (m/z = 170, Figure 11) réalise les transferts de charge et sa forme protonée (m/z = 171) permet les transferts de proton. Ces deux ions sont issus, selon Tubaro *et al.* [107], de la réaction d'un radical cation de phénol, produit dans la source par la réaction de benzène et d'oxygène, avec une molécule de benzène neutre.

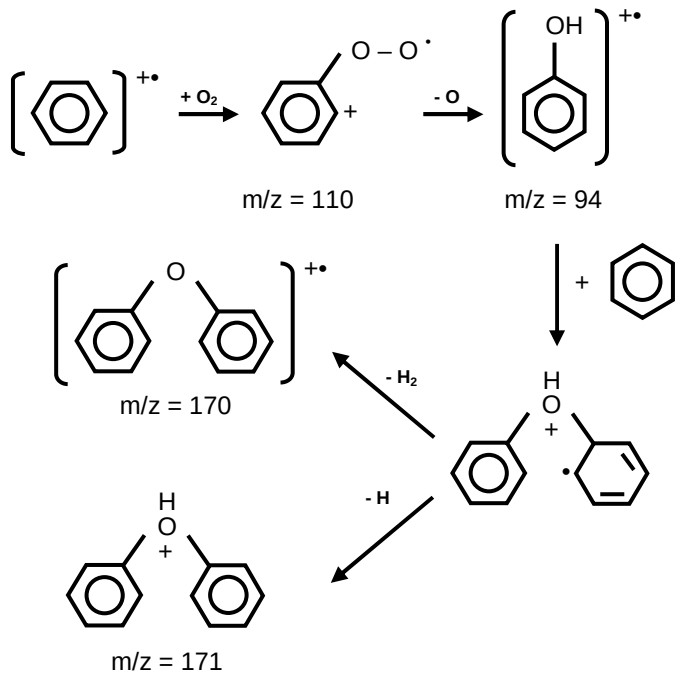


Figure 11 : mécanismes de formation des espèces réactionnelles de *m/z* = 170 et *m/z* = 171 à partir du benzène.

L'utilisation du toluène comme dopant produit des ions analogues à ceux responsables de l'ionisation dans le cas du benzène. Cependant, les divers ions produits lors de l'irradiation du toluène sont plus nombreux et de rapport masse sur charge plus élevé, rendant les mécanismes réactionnels du toluène pour l'ionisation des analytes encore inexpliqués à ce jour [107].
De plus, la quantité d'ions de toluène produite est directement proportionnelle à la quantité de dopant ajoutée (à concurrence de 10% de dopant), à la tension de la lampe appliquée (à concurrence de 0.8 mA appliqués) et inversement proportionnelle au débit de phase mobile [29;80].
Le toluène et, dans une moindre mesure l'acétone, favorisent l'ionisation des analytes par transfert de proton. Outre ce type de transfert, le toluène permet également le transfert de charge, mais de manière moins efficace [9;10].

A l'instar de la situation sans dopant, le MeOH permet un meilleur rendement d'ionisation que l'ACN, aussi bien en présence d'acétone que de toluène [80]. Avec le toluène, le transfert de proton est favorisé entre son ion ($[C_7H_8]^{+\bullet}$) et les clusters de MeOH et d'ACN formés dans la source car leurs PA sont plus élevées que celle de $[C_7H_8]^{+\bullet}$. L'ionisation des analytes s'en voit donc améliorée, le transfert de proton vers l'analyte étant effectué *via* ces clusters [34]. Les paramètres tels que la température du nébuliseur et le débit de phase mobile sont prépondérants dans le rendement d'ionisation [34]. La température du nébuliseur doit être optimisée avec soin, sachant que la taille des clusters lui est inversement proportionnelle. Le débit optimal de phase mobile se situe quant à lui typiquement entre 50 $\mu L \cdot min^{-1}$ et 100 $\mu L \cdot min^{-1}$ [11;82].
Des mélanges de dopants peuvent également être utilisés, mais le gain n'est pas significatif dans les conditions testées. Une première étude a conclu que le mélange optimal pour l'ionisation de PAH comprend 0.5% d'anisole dans le toluène [60] alors que la seconde n'a pas trouvé de bénéfice à l'ajout d'acétone dans le toluène pour l'ionisation de divers composés pharmaceutiques [52].
Enfin, l'utilisation d'un dopant n'est pas toujours bénéfique puisque, dans quelques rares cas, l'ajout de dopant peut même avoir un effet délétère sur l'ionisation [10;106].

Pour pallier la baisse d'ionisation en présence d'ACN dans la phase mobile, Kauppila *et al.* ont proposé l'anisole comme dopant [33]. Ce dernier n'ionisant les analytes que par transfert de charge, l'anisole est plus efficace que le toluène pour les composés de faibles PA et EI. En revanche, dans le cas d'analytes présentant des IE supérieures à celle de l'anisole, l'ionisation n'a pas lieu puisque le transfert de charge est impossible [33].

L'utilisation du THF et de l'hexafluorobenzène comme dopants n'est mentionnée dans la littérature qu'à titre anecdotique puisque seules deux études ne révélant aucun gain significatif sont parues sur le sujet [106;108].

1.2.4 APPI en mode négatif

L'ionisation en mode négatif se déroule selon trois mécanismes principaux [34], à savoir le transfert de charge (CX, (13)), la capture d'électron (EC, (14)) et le transfert de proton (déprotonation de l'analyte, (15)) :

$$M + C^- \rightarrow M^- + C \quad (13)$$

$$M + e^- \rightarrow M^- \quad (14)$$

$$M + C \rightarrow [M-H]^- + [C+H]^+ \quad (15)$$

Afin d'expliquer l'ionisation de certaines molécules, Song *et al.* ont récemment proposé un quatrième mécanisme appelé attachement d'anion (AA) (16) [95] :

$$M + A^- \rightarrow [M+A]^- \quad (16)$$

Lors de l'utilisation de la source PhotoMate, le mécanisme d'ionisation principal est la EC, puisqu'une grande quantité d'électrons lents est générée par l'irradiation de la surface métallique à l'entrée de la MS faisant face à la lampe [10;109]. L'oxygène naturellement présent dans l'air joue le rôle d'intermédiaire dans les mécanismes d'ionisation en mode négatif puisqu'il capture les électrons lents pour former $O_2^{\bullet-}$ [34;64]. Dans le cas le plus courant, $O_2^{\bullet-}$ ionise les analytes majoritairement par oxydation en $[M-H+O]^-$. En revanche, dans le cas de l'ionisation d'un acide, l'ionisation se produit par capture d'un proton par $O_2^{\bullet-}$, étant donné sa grande basicité en phase gazeuse [110]. De même, l'ajout d'acide ou de base dans la phase mobile diminue la formation de $M^{\bullet-}$ et des produits d'oxydation par la consommation des électrons lents [34;95;108]. En revanche, l'ajout d'un sel ne diminue que la production de $M^{\bullet-}$ sans entraver celle des produits d'oxydation [34;64].

1.2.5 L'APPI comparée à l'ESI et à l'APCI et sources mixtes

L'APPI apparaît comme étant une bonne alternative à l'ESI et l'APCI pour le couplage de techniques séparatives miniaturisées telles que la CE et la LC capillaire, puisque mieux adaptée aux bas débits. En termes d'application, l'APPI semble être plus complémentaire à l'ESI qu'à l'APCI étant donné sa capacité à ioniser des molécules plus apolaires que cette dernière. La palette des polarités balayée par l'ESI et l'APPI est donc plus vaste que dans le cas de l'ESI et de l'APCI. Au-delà de la comparaison de ces sources, l'idée de conjuguer leur utilisation a donné naissance à des sources mixtes permettant deux types d'ionisation au sein de la même interface (ESI / APCI, ESI / APPI ou APCI / APPI). Ces sources peuvent élargir le domaine d'ionisation durant une même séparation, abaisser les limites de sensibilité et améliorer la répétabilité [11].

La principale différence entre l'APCI et l'APPI réside dans les rendements d'ionisation en fonction du débit de phase mobile. En effet, l'APPI permet une ionisation optimale avec des débits de l'ordre de 50 à 100 $\mu L \cdot min^{-1}$, alors que l'APCI nécessite des débits plus importants (>1 $mL \cdot min^{-1}$). Cependant, Kauppila *et al.* [82] ont constaté que l'ionisation en APPI de composés de hautes PA n'était quasiment pas influencée par l'augmentation du débit de phase mobile, indiquant la plus grande susceptibilité des mécanismes de transfert de charge au débit de phase mobile. D'un point de vue pratique, l'APPI est également mieux adaptée à l'utilisation de solvants inflammables et ne présente pas les mêmes problèmes de baisse de signal au cours du temps dus au dépôt de carbone sur l'aiguille corona de l'APCI et sur la surface du cône [56]. En revanche, la lampe présente une durée de vie dépendante de la température opératoire déterminée comme étant supérieure à 500h à 300°C et supérieure à 2000h à température ambiante [69].

1.2.6 Effets matrice

Les effets d'altération du signal MS dus à la coélution de composés de la matrice n'ont été jusqu'à présent que peu approfondis en APPI. En effet, seules les deux études présentées ci-dessous ont étudié le sujet, les autres études ne l'ayant qu'au mieux mentionné.

Etant donné les mécanismes réactionnels régissant l'ionisation en APPI, cette dernière ne devrait *a priori* pas être entachée d'effets matrice. Cependant, des effets de suppression de signal peuvent survenir *après* l'ionisation des analytes, exactement de la même manière par laquelle ils ont été ionisés, à savoir par transfert de charge ou de proton vers d'autres composés également présents dans la source (solvants, composés endogènes *etc.*), l'analyte jouant ici le rôle d'intermédiaire pour l'ionisation de tierces molécules [10] :

$$[M+H]^+ + S \rightarrow M + [S+H]^+ \quad (17)$$

$$[M]^{\bullet +} + S \rightarrow [M-H]^{\bullet} + [S+H]^+ \quad (18)$$

Dans certains cas, des augmentations de signal ont été observées lors de l'ionisation d'analytes en présence de composés endogènes (voir article II). L'hypothèse proposée par les auteurs voudrait que les composés endogènes jouent un rôle de dopant, mais aucune confirmation n'a jusqu'à présent été apportée.

2. EVALUATION DES EFFETS MATRICE PROVENANT D'ECHANTILLONS PLASMATIQUES SUITE A LEUR EXTRACTION PAR SPE EN LIGNE

2.1 Introduction

Lors du développement de méthode, l'évaluation des effets matrice est nécessaire de manière à contrôler la sélectivité de la méthode. Deux techniques sont souvent utilisées à cet effet. La première est le principal moyen permettant l'évaluation des effets matrice lors de l'extraction en ligne. Le principe consiste en l'infusion post-colonne d'une solution de composés modèles (Figure 12) produisant un signal constant non-nul à la détection. La seconde technique a quant à elle fait l'objet d'une étude résumée au chapitre 4.

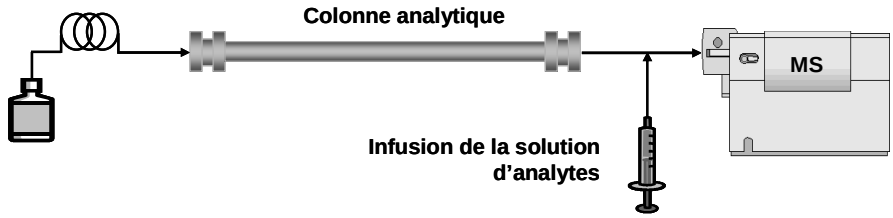


Figure 12 : schéma de l'infusion post-colonne.

Le principe consiste en l'extraction d'un échantillon de matrice blanche avec la méthode de préparation d'échantillon évaluée. La séparation est réalisée dans les conditions réelles d'analyse, permettant de localiser l'effet matrice sur le chromatogramme. Le signal constant généré par l'infusion de la solution d'analyte(s) est altéré lors de l'élution de composés endogènes résiduels. La caractérisation de cet effet est possible *via* trois paramètres, à savoir l'intensité (I), la durée (D) ainsi que la localisation sur le chromatogramme (L) (Figure 13).

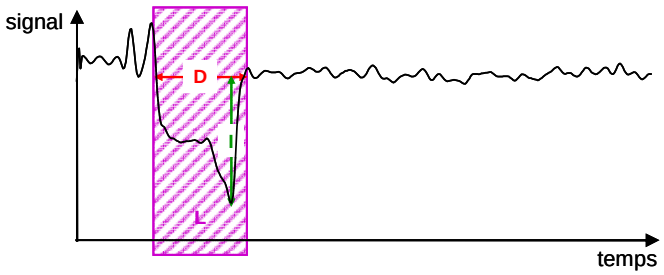


Figure 13 : exemple d'effet matrice négatif d'intensité I, de durée D et de localisation L.

L'information apportée par ce type d'expérience est complémentaire à celle obtenue lors de l'évaluation quantitative des effets matrice discutée au chapitre 4. En effet, la localisation permet de déterminer si le composé élué présente une rétention correspondant à la fenêtre de l'effet matrice. Le cas échéant, une modification des conditions de séparation peut éventuellement permettre de sortir le composé de la fenêtre de l'effet matrice et ainsi de s'en affranchir sans pour autant redévelopper la préparation d'échantillon.

2.2 Champs d'investigation

Le montage de l'infusion post-colonne a été employé dans le cadre de cette étude pour l'évaluation de la qualité de la préparation d'échantillons plasmatiques par SPE-LC-API-MS avec l'ESI, l'APCI et l'APPI comme sources d'ionisation. Les composés modèles sélectionnés sont la méthadone (MTD), le flunitrazepam (FLZ), la fluoxetine (FLX), leurs principaux métabolites respectivement le 2-éthyldiène-1,5-diméthyl-1,3-diphénylpyrrolidine (EDDP), le norflunitrazepam (NFLZ), la norfluoxetine (NFLX) ainsi que la vitamine D_3 (VD3) et un pesticide, le métalaxyl (MTX). Leur choix est basé sur les composés sélectionnés dans une précédente étude [111]. Quatre supports d'extraction ont quant à eux été évalués, à savoir un RAM (LiChrospher RP-4 ADS 25 x 2.0 mm I.D., d_p 25 µm), deux LPS (Oasis HLB 20 x 2.1 mm I.D., d_p 25 µm et Cyclone Turboflow HTLC 50 x 1.0 mm I.D., d_p 50 µm) et un monolithe (Chromolith Flash 25 x 4.6 mm I.D.). Le RAM a été testé au débit de chargement préconisé par le fabriquant (0.8 $mL \cdot min^{-1}$) ainsi qu'à un débit de 4.0 $mL \cdot min^{-1}$ comme pour les trois autres supports, puisque le diamètre des particules (25 µm) permet l'utilisation d'un tel débit sans perte de charge excessive [112]. Tous ces supports sont commercialisés pour l'injection directe d'échantillons biologiques. Dans cette optique, une simple dilution est effectuée et l'échantillon injecté. En revanche, la charge biologique étant importante, l'intérêt d'une PP préalable à l'injection a été également évaluée. Le choix de l'agent précipitant s'est porté sur l'ACN pour sa compatibilité directe avec le système d'analyse et pour son pouvoir précipitant (92% de protéines précipitées pour un rapport volumique 2:1) plus important que celui offert par le MeOH (90% de précipitation) et l'EtOH (88% de précipitation) dans les mêmes proportions. La PP par ajout d'acide ou de sel a été écartée car moins efficace et celle par ajout d'un ion métallique (typiquement du sulfate de zinc) pour des raisons d'élimination des déchets engendrés, malgré une élimination de 99% de la charge protéique [3]. Outre les échantillons plasmatiques dilués et précipités, un blanc (phase mobile) a également été injecté.

2.3 Traitement des données

De façon à ne tenir compte que des altérations de signal dues à l'élution de composés endogènes et à éliminer celles dues au montage (commutation de vanne, variation de phase mobile, *etc.*), le chromatogramme du blanc a été soustrait à celui des échantillons plasmatiques dilués et précipités.

L'effet matrice a été caractérisé pour chaque composé, chaque support d'extraction et chaque source *via* son intensité (I) et sa durée (D) (Figure 13). Le nombre de données générées étant conséquent (480), une analyse en composantes principales (PCA) a été menée de façon à mieux gérer l'information obtenue et déterminer les relations entre les variables et les individus (supports d'extraction avec ou sans PP). Les groupes formés ont été confirmés par une classification hiérarchique ascendante (HCA).

2.4 Résultats

La co-élution de matériel endogène entraîne une suppression du signal des analytes en ESI, alors qu'une augmentation de signal est observée en APCI et APPI. Cet effet dépend du composé analysé. Ainsi, la VD3 est l'analyte le plus sujet à ces effets alors que le MTX et le FLZ sont par exemple peu influencés. De manière générale, le rendement d'ionisation et la susceptibilité aux effets matrice sont décroissants dans l'ordre ESI - APCI - APPI. L'adaptation du choix de la source d'ionisation à une situation donnée devra donc être fait en considérant le meilleur compromis entre les deux facteurs précités.

Concernant le prétraitement de l'échantillon, la dilution présente des effets matrice quelque soit le support d'extraction, la source et le composé évalués. L'ESI est plus influencée, puisque des effets matrice ont été observés dans tous les cas. De plus, le support d'extraction LiChrospher ADS induit moins d'effets matrice après l'injection (à 0.8 $mL \cdot min^{-1}$) de plasma dilué que les autres supports testés à 4.0 $mL \cdot min^{-1}$. L'utilisation de l'Oasis HLB a, quant à elle, entraîné une augmentation de la perte de charge sur la colonne d'environ 1-2 bars par injection, probablement dû au débit de chargement trop faible par rapport aux dimensions de la colonne (20 x 2.1 mm I.D.). En effet, un débit de chargement de 16 $mL \cdot min^{-1}$ aurait été probablement plus adapté à l'élimination des protéines, mais ce débit était incompatible avec le système HPLC utilisé.

En revanche, lors d'une PP de l'échantillon préalable à l'injection, les effets matrice sont diminués dans 70% des cas en ESI et dans 83% des cas en APCI, alors qu'ils sont totalement éliminés dans 100% des cas en APPI.

La Figure 14 représente la PCA menée sur la totalité des 480 valeurs correspondant à l'intensité ainsi qu'à la durée des effets matrice rencontrés sur la totalité des composés modèles. Toutes les variables sont bien représentées sur les deux premiers axes des composantes principales (PC) expliquant plus de 80% de l'information totale. Les deux flèches ont été ajoutées de manière à clarifier la lecture du graphique et correspondent à des effets matrice croissants en termes d'intensité et de durée. Deux groupes sont clairement distingués, correspondant au prétraitement de l'échantillon. Le groupe de droite correspond aux injections de plasma dilué, alors que celui de gauche comprend les supports ayant bénéficié de l'injection de plasma précipité, confirmant l'observation faite précédemment concernant la diminution des effets matrice lors d'une PP préalable

à l'injection. De plus, la PP permet d'homogénéiser la qualité de l'échantillon préparé, quelque soit le support utilisé.

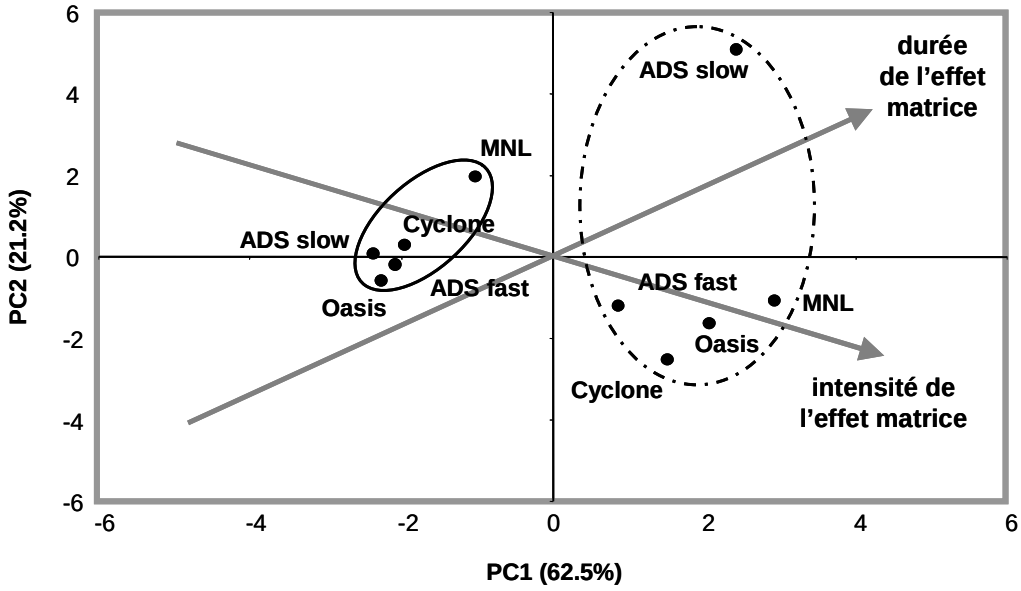


Figure 14 : PCA globale. Pointillés : plasma dilué. Ligne continue : plasma précipité.

2.5 Conclusion

Les supports d'extraction testés ne sont que peu ou pas adaptés à l'injection directe de plasma, démontrant dans la plupart des cas d'importants effets matrice. En revanche, dès lors qu'une PP est effectuée préalablement à l'injection, les effets matrice sont drastiquement diminués voire totalement éliminés dans le cas de l'utilisation de l'APPI. Le choix du support d'extraction devient alors secondaire puisque tous présentent des performances similaires en terme de propreté d'échantillon. L'APPI est la source la moins affectée par les effets matrice, suivie de l'APCI et de l'ESI. Finalement, notons qu'une séparation chromatographique permettant la séparation des analytes et des éventuels composés endogènes résiduels est indispensable de manière à éviter tout problème de quantification.

3. SELECTION DE COMPOSES REPRESENTATIFS DANS LA MISE AU POINT D'EXTRACTIONS SUR SUPPORT SOLIDE

3.1 Introduction

L'analyse d'une grande quantité de substances dans les matrices biologiques est requise dans de nombreux domaines tels que le *therapeutic drug monitoring*, la lutte anti-dopage, les sciences forensiques, la toxicologie, etc. Lors des analyses de routine, l'utilisation d'un nombre restreint de méthodes, permettant la détermination d'un grand nombre de substances, est généralement préférée. En LC-MS, l'étape de préparation d'échantillon est incoutournable lors de l'analyse de matrices biologiques pour limiter le bouchage de la colonne et les effets d'altération du signal MS. Le développement de la préparation d'échantillon est rendu plus fastidieux lorsque de nombreuses substances sont impliquées. C'est pourquoi, l'article III résumé dans ce paragraphe propose une solution pour réduire le temps et le nombre d'expériences requis pour le développement de méthode, au moyen de substances représentatives sélectionnées sur la base de leurs propriétés physico-chimiques déterminées pratiquement.

3.2 Choix du support d'extraction

La première étape a consisté à déterminer la phase SPE la mieux adaptée à la problématique parmi un choix de cinq supports présentant les principales chimies de phases disponibles. Une phase de rétention hydrophile-hydrophobe (Oasis HLB), deux échangeurs de cations (Oasis MCX; Oasis WCX) et deux échangeurs d'anions (Oasis MAX; Oasis WAX) ont ainsi été testés. Un mélange standard des trente-quatre composés d'intérêt a été extrait sur chaque support à l'aide une méthode générique et le taux de récupération (recouvrement) pour chaque analyte dans chaque phase (chargement, lavage, élution) a été déterminé. Le support le moins sélectif, à savoir l'Oasis HLB, n'a pas fourni de résultats satisfaisants du fait de la perte des bases au chargement à pH acide et neutre et des acides de faible polarité à pH basique. En revanche, l'Oasis MCX a présenté les meilleures rétentions au chargement (pH acide), au lavage et une élution totale (Tableau 5) de la majorité des composés. Ce support a donc été maintenu pour la suite de l'étude.

Tableau 5 : recouvrements calculés pour chaque analyte extrait sur Oasis MCX avec chargement à pH acide.

compound	load	wash	elute 1	elute 2
acébutolol	0%	0%	0%	98%
acétazolamide	0%	0%	35%	0
adrafinil	0%	0%	99%	1%
aténolol	0%	0%	31%	69%
bendrofluméthiazide	0%	0%	42%	4%
benzoylecgonine	23%	0%	3%	74%
bethaméthasone	0%	0%	100%	0%
bumétanide	0%	0%	91%	2%
canrénone	0%	0%	100%	0%
cartéolol	0%	0%	0%	98%
celiprolol	0%	1%	0%	99%
chlorothiazide	0%	0%	95%	0%
chlortalidone	0%	0%	99%	1%
clopamide	0%	0%	94%	6%
dexaméthasone	0%	0%	100%	0%
dichlorphénamide	0%	0%	83%	1%
esmolol	0%	0%	0%	85%
acide éthacrynique	27%	0%	70%	2%
finastéride	0%	0%	97%	3%
furosémide	0%	0%	97%	3%
gestrinone	0%	0%	80%	2%
hydrochlorothiazide	0%	0%	90%	2%
indapamide	0%	0%	98%	2%
métipranolol	0%	0%	0%	100%
métolazone	0%	0%	91%	1%
métoprolol	0%	0%	%0	100%
modafinil	4%	0%	70%	1%
nadolol	0%	0%	0%	100%
pirétanide	0%	0%	75%	0%
probénécide	0%	0%	88%	2%
sotalol	0%	0%	0%	100%
strychnine	0%	0%	1%	93%
torasémide	0%	0%	0%	100%
xipamide	0%	0%	97%	3%

Les résultats obtenus confirment que les composés acides et neutres sont retenus par un mécanisme de type hydrophobe et élués en présence de MeOH. Les bases (protonées) sont quant à elles retenues selon deux interactions, hydrophobe et ionique. Elles nécessitent du MeOH basique pour leur élution.

3.3 Sélections des substances représentatives

Le principe du développement de la méthode est basé sur l'utilisation d'un nombre restreint de composés pour l'optimisation des conditions de chacune des étapes de la SPE, diminuant ainsi le nombre d'expériences et le temps nécessaire au traitement des données. Une attention toute particulière a été portée à la sélection de ces composés. L'utilisation d'un petit nombre d'analytes ne peut être envisagée que si les composés sélectionnés sont représentatifs des autres molécules en terme de comportement SPE. La solution consistant à sélectionner les composés dans des classes pharmaceutiques n'est pas adéquate puisque diverses familles chimiques présentant des comportements SPE variés peuvent être représentées. Par ailleurs, les valeurs de pKa, logD et logP des analytes ne sont pas toujours disponibles dans la littérature ou ne reflètent pas la réalité (valeurs calculées). En revanche, les informations apportées par l'évaluation pratique du comportement SPE sont plus fiables. Les données obtenues pour le choix du support ont été utilisées pour la sélection des composés représentatifs. Compte tenu du grand nombre de valeurs (544), une classification ascendante hiérarchique (HCA) a été menée et a permis de dégager quatre groupes de composés présentant des comportements SPE similaires, correspondant à diverses classes chimiques, à savoir acides (7), acides faibles (10), neutres (6) et bases (11) (Figure 15).

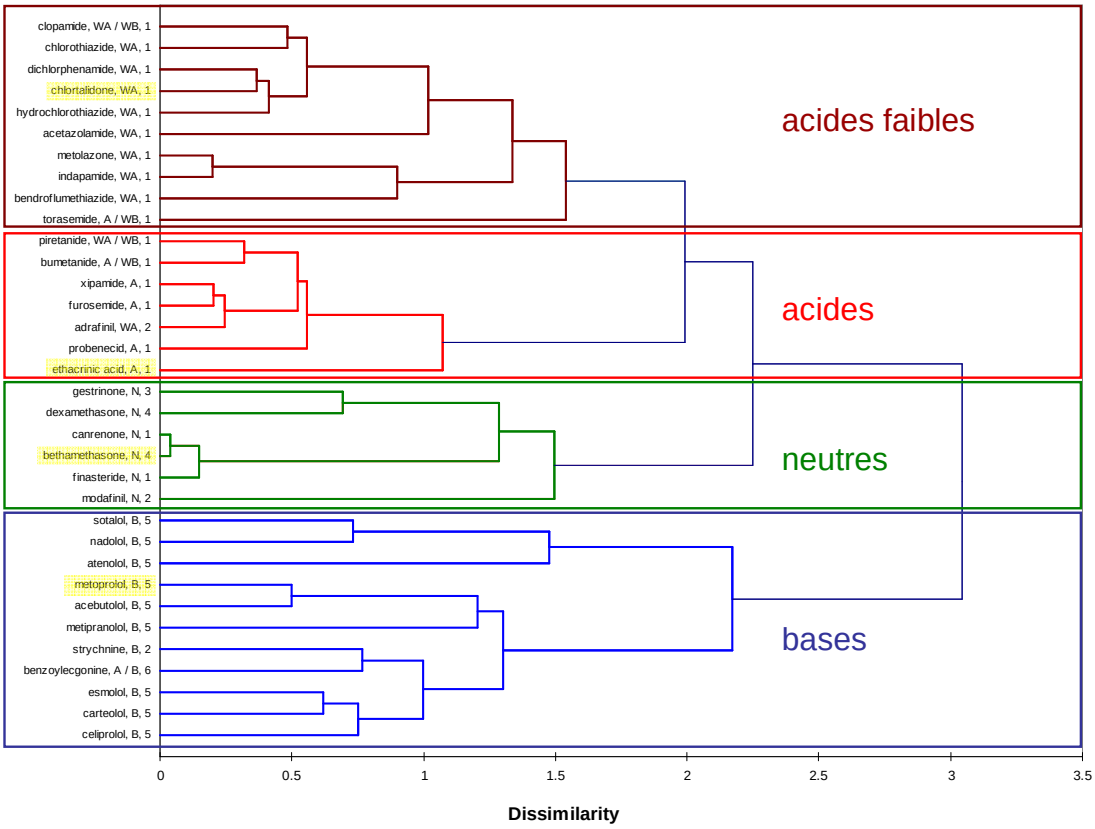


Figure 15 : dendrogramme de dissimilarité du comportement SPE des 34 composés. A = acide, B = base, N = neutre, WA = acide faible, WB = base faible, 1 = diurétique, 2 = stimulant, 3 = anabolisant, 4 = corticostéroïde, 5 = β-bloquant, 6 = narcotique. Les composés représentatifs sont surlignés.

Les groupes formés comprennent des composés de classes pharmaceutiques différentes, démontrant que celles-ci ne sont pas suffisantes pour la détermination du comportement SPE. Ainsi, l'évaluation pratique du comportement SPE est nécessaire pour le choix des composés représentatifs puisque, par exemple, la benzoylecgonine se comporte comme une base, information inaccessible *via* la simple observation des valeurs physico-chimiques.

Parmi chacun des groupes, le choix des analytes représentatifs a été effectué en se basant sur les valeurs de logP et logD. En effet, l'acide éthacrynique a par exemple été sélectionné comme composé représentatif des acides pour ses valeurs moyennes de logP (3.38) et logD (2.9, -0.8 et -1.5 à pH1, pH7 et pH10, respectivement) (Tableau 6). La betaméthasone, la chlortalidone et le métoprolol ont été choisis selon les mêmes critères pour les neutres, les acides faibles et les bases, respectivement.

Tableau 6 : classes thérapeutiques et valeurs de pKa, logD et logP pour chaque composé (gras : composés représentatifs). Les valeurs ont été calculées avec le programme Advanced Chemistry Development (ACD/Labs) V8.14 pour Solaris (© 1994-2006 ACD/Labs)

composé	classe thérapeutique	pKa acide	pKa basique	log D			logP
				pH1	pH7	pH10	
acébutolol	β-bloquant	13.8	9.1	-0.5	0.5	2.5	2.6
acétazolamide	diurétique	7.4	-	-0.3	-0.4	-3.8	-0.2
adrafinil	stimulant	8.2	-	1.0	1.0	-0.8	1.0
aténolol	β-bloquant	13.9	9.2	-3.0	-2.0	0.0	0.1
bendrofluméthiazide	diurétique	8.6	-	2.0	2.0	-0.1	2.0
benzoylecgonine	narcotique	3.5	10.8	-0.4	0.2	0.2	2.7
bethaméthasone	corticostéroïde	12.1	-	2.1	2.1	2.2	2.0
bumétanide	diurétique	3.2	4.5	0.5	-0.3	-1.5	2.7
canrénone	diurétique	-	-	3.0	3.0	3.0	2.9
cartéolol	β-bloquant	13.8	9.1	-1.4	-0.4	1.6	1.6
celiprolol	β-bloquant	13.8	9.1	-0.3	0.8	2.8	2.8
chlorothiazide	diurétique	9.2	-	-0.2	-0.2	-1.1	-0.1
chlortalidone	diurétique	9.6	-	-0.7	-0.7	-1.4	-0.7
clopamide	diurétique	9.4	4.1	-1.2	1.6	0.9	1.5
dexaméthasone	corticostéroïde	12.1	-	1.9	1.9	1.9	1.8
dichlorphénamide	diurétique	9.0	-	0.9	0.9	-0.9	0.9
esmolol	β-bloquant	13.9	9.2	-1.2	-0.2	1.9	1.9
acide éthacrynique	diurétique	2.8	-	3.4	-0.5	-0.7	3.3
finastéride	diurétique	-	-	3.2	3.2	3.2	3.2
furosémide	diurétique	3.0	-	2.9	-0.8	-1.5	2.9
gestrinone	anabolisant	-	-	3.3	3.3	3.3	3.3
hydrochlorothiazide	diurétique	9.0	-	-0.1	-0.1	-1.8	-0.1
indapamide	diurétique	9.4	-	2.1	2.1	1.2	2.1
métipranolol	β-bloquant	13.9	9.2	-0.4	0.5	2.6	2.6
métolazone	diurétique	10.0	-	3.2	3.2	2.9	3.1
métoprolol	β-bloquant	13.9	9.2	-1.3	-0.3	1.7	1.7
modafinil	stimulant	-	-	1.4	1.4	1.4	1.4
nadolol	β-bloquant	13.9	9.2	-1.8	-0.8	1.2	1.2
pirétanide	diurétique	10.2	4.3	-0.0	-1.2	-2.4	1.8
probénécide	diurétique	3.7	-	3.3	0.1	-0.8	3.3
sotalol	β-bloquant	9.6	9.2	-2.8	-1.8	-0.3	0.3
strychnine	stimulant	-	8.3	-1.4	0.3	1.6	1.6
torasémide	diurétique	3.1	4.8	0.7	0.5	-0.9	3.1
xipamide	diurétique	5.1	-	4.0	2.1	0.5	4.0

3.4 Optimisation de la méthode SPE

Chaque étape de l'extraction a ensuite été optimisée avec ces quatre analytes en termes de volume, de composition et de pH de phase mobile. La méthode d'extraction optimisée nécessite le chargement de 1.0 mL d'échantillon préalablement dilué 1:1 avec une solution de HCl 240 mM. Le lavage est effectué avec 1.0 mL d'un mélange HCl 120mM / MeOH 90:10, suivi d'une élution avec 250 µL de MeOH. La seconde élution est réalisée avec le même volume de MeOH + 5% NH_4OH. Une fois ces conditions de chargement, lavage et élution déterminées, la méthode a été évaluée en triplicata par l'extraction d'un standard aqueux contant les trente-quatre composés (illustration 1, Figure 16a). Les recouvrements ont été calculés en comparaison à un standard aqueux non extrait (illustration 2, Figure 16a). Ils étaient dans tous les cas compris entre 80% et 110% avec des CV inférieurs à 10%, témoignant de la bonne représentativité des quatre composés sélectionnés.

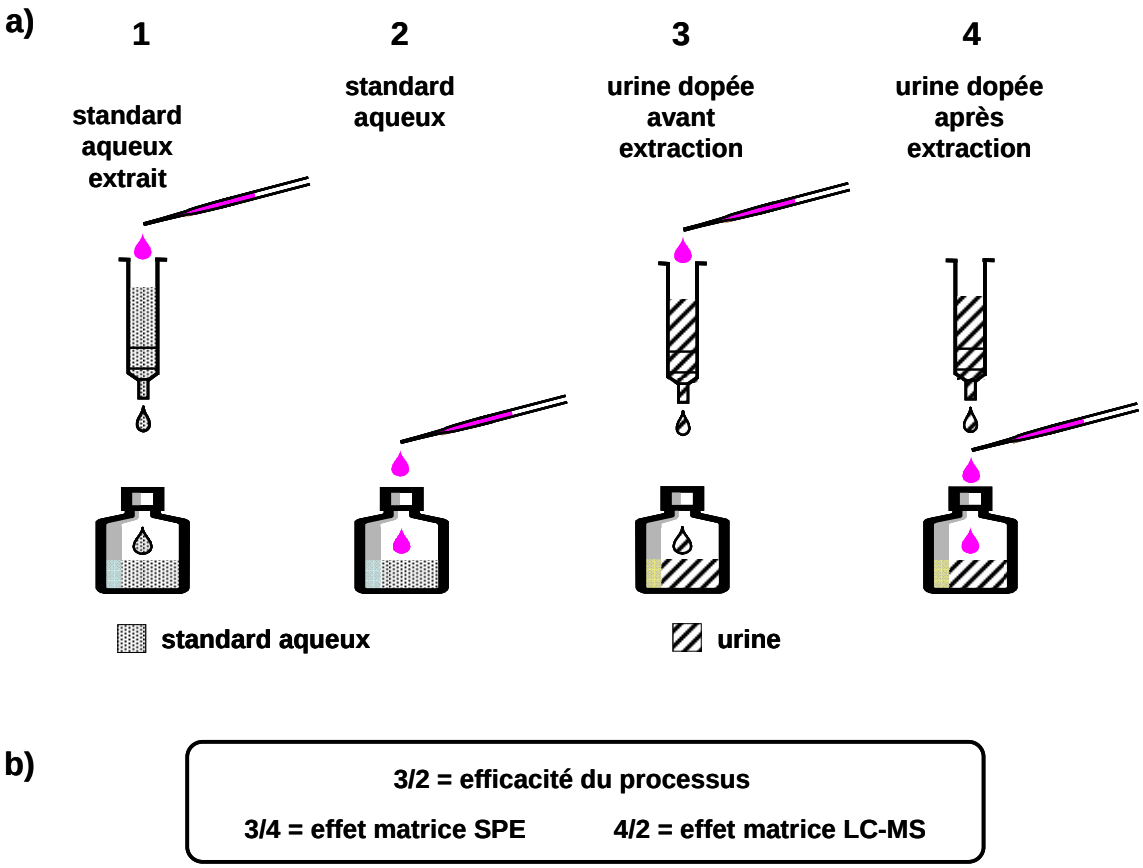


Figure 16 : représentation des quatre expériences nécessaires à l'évaluation complète des effets matrice.

3.5 Effets matrice

L'évaluation des effets matrice entreprise dans cette étude est basée sur la méthode proposée par Matuszewski *et al.* [113] permettant de déterminer leur origine. L'efficacité du processus (somme de tous les effets rencontrés lors de l'analyse complète) est évaluée par la comparaison d'un échantillon d'urine dopée avant la préparation (illustration 3, Figure 16a) avec un standard aqueux (illustration 2, Figure 16a). Les efficacités du processus étaient comprises entre 10% et 97% avec des CV < 27% (Tableau 7), indiquant une interférence de la matrice lors du processus.

Tableau 7 : efficacité du processus, effet matrice sur la MS et effet de la matrice sur la SPE avec les CV de répétabilité.

compound	efficacité du processus (CV)	MS (CV)	SPE (CV)
acébutolol	59% (2%)	65% (3%)	90% (4%)
acétazolamide	26% (10%)	28% (6%)	92% (10%)
adrafinil	54% (3%)	55% (2%)	98% (6%)
aténolol	48% (6%)	53% (3%)	91% (7%)
bendroflumethiazide	69% (6%)	72% (2%)	96% (5%)
benzoylecgonine	46% (5%)	49% (8%)	91% (1%)
betaméthasone	77% (27%)	72% (33%)	106% (4%)
bumétanide	82% (1%)	86% (5%)	95% (7%)
canrénone	69% (11%)	71% (10%)	99% (5%)
cartéolol	64% (4%)	64% (9%)	100% (7%)
celiprolol	71% (3%)	79% (5%)	90% (4%)
chlorothiazide	24% (8%)	26% (5%)	91% (4%)
chlorthalidone	75% (2%)	65% (10%)	114% (10%)
clopamide	35% (2%)	38% (4%)	91% (3%)
dexaméthasone	79% (3%)	80% (1%)	98% (3%)
dichlorphénamide	86% (5%)	78% (4%)	109% (6%)
esmolol	10% (12%)	9% (4%)	109% (11%)
acide éthacrynique	52% (8%)	56% (6%)	93% (10%)
finastéride	73% (11%)	75% (12%)	94% (5%)
furosémide	83% (1%)	98% (4%)	85% (3%)
gestrinone	74% (10%)	74% (15%)	100% (5%)
hydrochlorothiazide	62% (2%)	55% (6%)	113% (4%)
indapamide	97% (16%)	94% (13%)	98% (7%)
métipranolol	56% (16%)	61% (5%)	93% (12%)
métolazone	33% (20%)	26% (15%)	111% (10%)
métoprolol	27% (7%)	30% (3%)	89% (7%)
modafinil	96% (11%)	83% (10%)	107% (15%)
nadolol	54% (10%)	53% (2%)	97% (10%)
pirétanide	51% (25%)	46% (8%)	93% (9%)
probénécide	93% (3%)	100% (3%)	93% (10%)
sotalol	58% (15%)	51% (6%)	109% (9%)
strychnine	36% (6%)	39% (15%)	94% (9%)
torasémide	57% (8%)	66% (7%)	85% (5%)
xipamide	68% (5%)	67% (2%)	101% (7%)

De manière à déterminer si le problème identifié est dû à un effet de la matrice sur l'extraction, un échantillon de matrice dopé après extraction (illustration 4, Figure 16a) est comparé à l'échantillon d'urine dopé avant extraction. L'effet matrice lors de l'analyse est mis en évidence par la comparaison d'un échantillon de matrice dopé après extraction avec un standard aqueux.

Etant donné qu'une faible efficacité du processus peut être due à diverses combinaisons de l'effet de la matrice sur la SPE et sur la MS, une classification des divers cas possibles a été proposée. Sachant que l'effet de la matrice sur l'extraction ne peut être que négatif (symbolisé par -1) ou nul (symbolisé par 0) et négatif (-1), nul (0) ou positif (+1) sur l'analyse, seules huit combinaisons sont alors envisageables (Tableau 8).

Tableau 8 : résumé des cas d'effets matrice possibles (effet négatif (-1), pas d'effet (0), effet positif (+)).

efficacité du processus	-1	-1	-1	-1	0	0	1	1
LC-MS	-1	-1	0	1	0	1	1	1
SPE	-1	0	-1	-1	0	-1	-1	0
cas	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**

Le cas numéro 4 correspond par exemple à deux effets matrice combinés et de sens opposé, plus intense sur la SPE (-1) que sur l'analyse (+1), entraînant une faible efficacité du processus (-1). Cette classification a ensuite été utilisée pour déterminer à quel cas d'effet matrice appartenait chaque composé. La limite déterminant un effet significatif (attribution de -1, 0 ou +1)a été fixée à ± 10%. Les résultats sont reportés dans le Tableau 9.

Tableau 9 : cas de l'effet matrice pour chaque analyte (gras : composés représentatifs).

composé	cas	groupe (voir Figure 15)
acébutolol	1	B
acétazolamide	2	WA
adrafinil	2	A
aténolol	2	B
bendroflumethiazide	2	WA
benzoylecgonine	2	B
betaméthasone	2	N
bumétanide	2	A
canrénone	2	N
cartéolol	2	B
celiprolol	2	B
chlorothiazide	2	WA
chlorthalidone	2	WA
clopamide	2	WA
dexaméthasone	2	N
dichlorphénamide	2	WA
esmolol	2	B
acide éthacrynique	2	A
finastéride	2	N
furosémide	3	A
gestrinone	2	N
hydrochlorothiazide	2	WA
indapamide	5	WA
métipranolol	2	B
métolazone	2	WA
métoprolol	1	B
modafinil	5	N
nadolol	2	B
pirétanide	2	A
probénécide	5	A
sotalol	2	B
strychnine	2	B
torasémide	1	WA
xipamide	2	A

Les résultats présentés dans le Tableau 15 démontrent l'absence de corrélation entre la famille chimique du composé et le type d'effet matrice subit, indiquant que l'utilisation des composés représentatifs pour l'évaluation des effets matrice n'est pas possible.

3.6 Conclusion

La méthode proposée dans cette étude a permis le développement pour l'extraction par SPE de trente-quatre composés dans l'urine. La sélection des composés représentatifs à l'aide d'une classification ascendante hiérarchique (HCA) est basée sur les données du choix du support. Elle a permis de générer des groupes de molécules présentant des comportements SPE similaires. Pour chaque groupe formé, un composé représentatif a été employé pour l'optimisation du protocole d'extraction sur le support, permettant ainsi de diminuer le temps nécessaire aux manipulations et au traitement de données. La procédure optimisée à l'aide de ces quatre composés est parfaitement adaptée à l'extraction de la totalité des trente-quatre composés de l'étude, démontrant que la méthode choisie permet effectivement l'optimisation du protocole avec un nombre restreint d'analytes. En revanche, ces derniers ne sont pas représentatifs des effets matrice, nécessitant une évaluation individuelle de chaque composé.

4. CARACTERISATION ET CLASSIFICATION DES EFFETS MATRICE LORS DE L'ANALYSE D'ECHANTILLONS BIOLOGIQUES

4.1 Introduction

Durant le développement et la validation d'une méthode analytique, la maîtrise de la sélectivité est essentielle, raison pour laquelle l'évaluation des effets matrices est nécessaire.
Parmi les principaux outils d'identification des effets matrice, deux ont été utilisés au cours de ce travail de thèse. Le premier, dont l'utilisation a été exposée au chapitre 2, a été décrit dans une étude présentée en 1999 par Bonfiglio *et al.* [114] et propose une évaluation des effets matrice par infusion post-colonne, ne donnant qu'une information qualitative. Le second outil a, quant à lui, fait l'objet d'une étude publiée en 2003 par Matuszewski *et al.* [113] introduisant l'examen quantitatif des effets matrice *via* la comparaison des réponses obtenues pour les analytes dans divers échantillons. En revanche, aucune étude n'a été menée à ce jour sur la classification rigoureuse de ces effets. L'article IV résumé ici se propose donc de caractériser et répertorier ces effets de manière exhaustive en se basant sur les travaux de Matuszewski *et al.*

4.2 Evaluation quantitative des effets matrice

Le mode d'évaluation des effets matrice proposé par Matuszewski *et al.* fait intervenir trois notions. En effet, l'*efficacité du processus* (PE) rend compte du recouvrement de l'analyte sur la méthode complète. Il correspond donc à la combinaison du *recouvrement d'extraction* (RE) de la préparation d'échantillon en présence de la matrice et de l'*effet matrice sur l'analyse* (ME) (Figure 17).

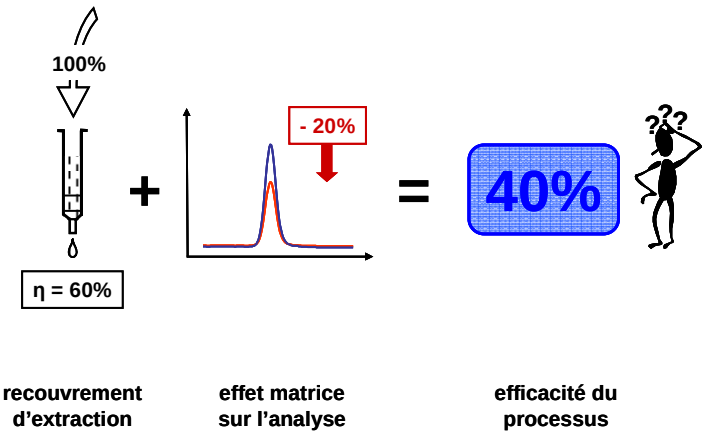


Figure 17 : décomposition schématisée de l'efficacité du processus.

Le PE est évaluée *via* le recouvrement d'un échantillon de matrice dopée puis extraite (***c***, Figure 18) rapporté à un standard aqueux (a, Figure 18). Le RE est quant à lui déterminé par le recouvrement entre l'échantillon ***c*** et un échantillon de matrice blanche dopée après extraction (***b***, Figure 18). Finalement, le ME est mis en évidence par le recouvrement entre les échantillons ***a*** et ***b***.

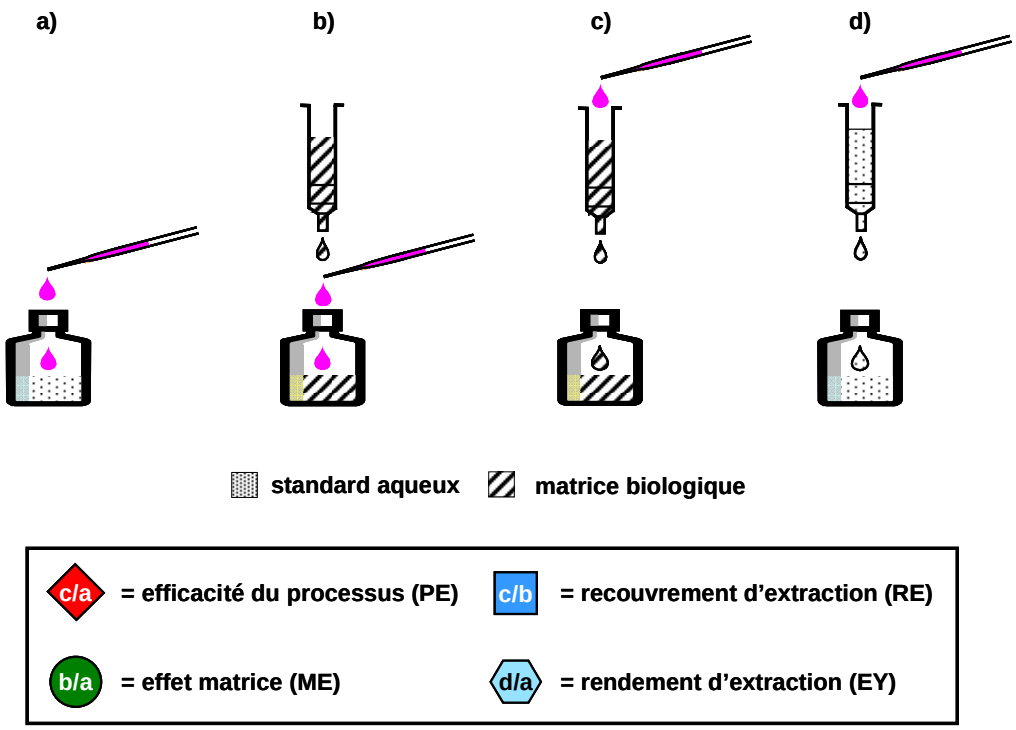


Figure 18 : illustration des quatre échantillons nécessaires à l'identification d'un effet matrice a) standard aqueux b) matrice blanche extraite puis dopée c) extraction de matrice dopée et d) standard aqueux extrait.

Dans le cas de l'utilisation d'une préparation d'échantillon générique, le RE peut être entaché par de mauvaises performances de la préparation d'échantillon elle-même. La notion de *rendement d'extraction* (EY) a donc été introduite dans cette étude et estimée *via* le rapport des aires des échantillons ***d*** et ***a***.

4.3 Description des cas

Le PE peut être inférieure, proche ou supérieure à 100%, symbolisé par -1, 0 et +1, respectivement, puisqu'elle correspond à la combinaison du RE et du ME. En effet, le ME peut lui aussi être inférieur, proche ou supérieur à 100% puisqu'aussi bien des suppressions que des augmentations de signal sont couramment rencontrées en MS. Les RE et EY ne peuvent quant à eux raisonnablement être

qu'inférieurs ou proches de 100%. Sur la base de ces considérations, les huit combinaisons possibles sont résumées dans le Tableau 10.

Tableau 10 : résumé des cas possibles

PE	**-1**				**0**		**+1**	
ME	**-1**	**-1**	**0**	**+1**	**0**	**+1**	**+1**	**+1**
RE	**-1**	**0**	**-1**	**-1**	**0**	**-1**	**-1**	**0**
	PE^{-}_{-}	**PE^{-}_{0}**	**PE^{0}_{-}**	**PE^{+}_{-}**	**PE^{o}_{o}**	**PE^{+}_{-}**	**PE^{+}_{-}**	**PE^{+}_{0}**

De manière à simplifier la dénomination de chaque cas, la convention suivante a été proposée : le signe en exposant indique le ME et le signe en indice correspond au RE.
Un PE inférieur à 100% peut être dû à diverses combinaisons ; un effet de la matrice sur l'une ou l'autre des étapes (PE^{-}_{0} and PE^{0}_{-}) et un effet combiné de la matrice sur l'extraction et l'analyse , synergique (PE^{-}_{-}) ou antagoniste (PE^{+}_{-}). Un PE proche de 100% ne peut lui être dû qu'à deux combinaisons ; une absence d'effet matrice sur le RE et le ME (PE^{0}_{0}) ou un effet antagoniste d'intensité comparable (PE^{+}_{-}). Finalement, un PE supérieur à 100% ne peut lui aussi être dû qu'à deux combinaisons ; une augmentation de signal partiellement compensée ou non par un RE inférieur à 100% (PE^{+}_{-} and PE^{+}_{0}, respectivement). Les cinq cas présentant un RE inférieur à 100% peuvent se diviser en deux modalités, à savoir un RE inférieur à 100% partiellement ou non dû à un mauvais EY.

Une arborescence a également été mise en place pour simplifier l'attribution de la typologie de l'effet matrice rencontré (Figure 19). L'identification de l'effet matrice est réalisée par simple cheminement sur le tracé en choisissant l'embranchement correspondant au résultat obtenu pour la contribution en question.

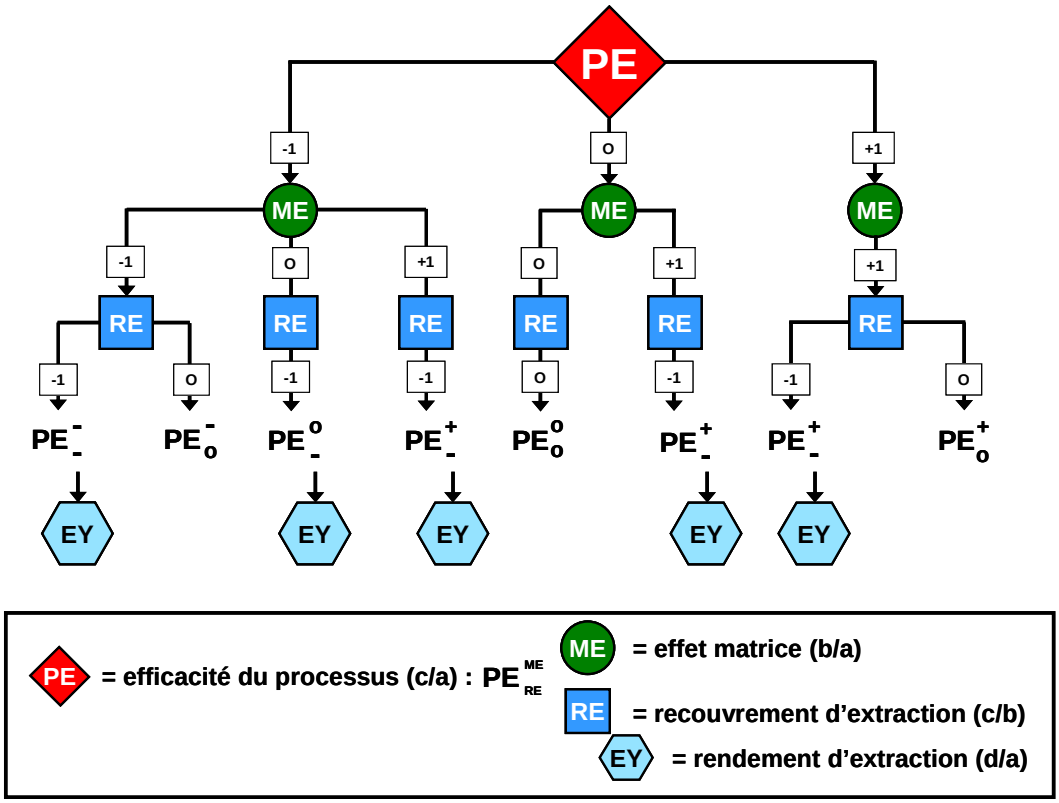


Figure 19 : schéma décisionnel permettant de déterminer le type d'effet matrice rencontré lors de l'emploi d'une méthode optimisée.

4.4 Application

De manière à contrôler la validité de la classification proposée, 199 composés ont été analysés avec trois procédures différentes utilisant trois supports d'extraction, deux matrice et trois appareils LC-ESI-MS. Les PE, ME, RE et EY ont été calculés pour chaque composé. Une limite raisonnable de ± 10% a été choisie pour distinguer les cas -1 et +1 de 0.

Tableau 11 : valeurs de PE, ME, RE et EY calculées pour chacun des 199 analytes.

composé	matrice	appareillage	PE	ME	RE	EY
Alprazolam	plasma	LC-MS/MS	45%	49%	90%	82%
Amfepramone	plasma	LC-MS/MS	93%	92%	103%	101%
Amisulpride	plasma	LC-MS/MS	93%	132%	74%	91%
Amitriptyline	plasma	LC-MS/MS	54%	61%	91%	100%
Amphetamine	plasma	LC-MS/MS	78%	91%	86%	89%
Aripirazole	plasma	LC-MS/MS	80%	102%	78%	99%
Atenolol	plasma	LC-MS/MS	44%	92%	48%	34%
Bisoprolol	plasma	LC-MS/MS	101%	99%	102%	96%
Bromazepam	plasma	LC-MS/MS	79%	89%	91%	91%
Buprenorphine	plasma	LC-MS/MS	89%	109%	84%	96%
Bupropion	plasma	LC-MS/MS	109%	134%	83%	91%

Chlordiazepoxide	plasma	LC-MS/MS	93%	91%	103%	95%
Chloroquine	plasma	LC-MS/MS	107%	129%	84%	88%
Chlorprothixène	plasma	LC-MS/MS	36%	53%	71%	90%
Citalopram	plasma	LC-MS/MS	90%	109%	82%	94%
Clobazam	plasma	LC-MS/MS	38%	40%	94%	66%
Clomipramine	plasma	LC-MS/MS	62%	95%	65%	101%
Clonazepam	plasma	LC-MS/MS	34%	41%	83%	86%
Clonidine	plasma	LC-MS/MS	95%	128%	76%	100%
Clotiapine	plasma	LC-MS/MS	29%	33%	90%	105%
Clozapine	plasma	LC-MS/MS	92%	104%	89%	82%
Cocaïne	plasma	LC-MS/MS	92%	97%	96%	93%
Codéine	plasma	LC-MS/MS	85%	105%	81%	16%
Cotinine	plasma	LC-MS/MS	2%	32%	6%	8%
Cyclizine	plasma	LC-MS/MS	95%	109%	88%	94%
Desipramine	plasma	LC-MS/MS	47%	45%	105%	119%
Desmethyl-Chlordiazepoxide	plasma	LC-MS/MS	3%	7%	47%	7%
Dextrometrophane	plasma	LC-MS/MS	77%	89%	86%	99%
Dimetindène	plasma	LC-MS/MS	46%	65%	71%	109%
Diphenhydramine	plasma	LC-MS/MS	84%	111%	77%	56%
Duloxetine	plasma	LC-MS/MS	29%	42%	75%	71%
EDDP	plasma	LC-MS/MS	76%	88%	85%	83%
Flumazenil	plasma	LC-MS/MS	88%	110%	81%	93%
Flunitrazepam	plasma	LC-MS/MS	28%	43%	66%	72%
Fluoxetine	plasma	LC-MS/MS	53%	63%	85%	58%
Fluphenazine	plasma	LC-MS/MS	73%	84%	87%	101%
Fluvoxamine	plasma	LC-MS/MS	74%	103%	72%	98%
Haloperidol	plasma	LC-MS/MS	89%	96%	93%	96%
Héroïne	plasma	LC-MS/MS	107%	248%	44%	93%
9-Hydroxy-Risperidone	plasma	LC-MS/MS	92%	107%	86%	83%
Imipramime	plasma	LC-MS/MS	36%	73%	50%	104%
Indométhacine	plasma	LC-MS/MS	24%	55%	44%	57%
Lamotrigine	plasma	LC-MS/MS	89%	116%	77%	102%
Levopromazine	plasma	LC-MS/MS	14%	26%	56%	59%
Lidocaïne	plasma	LC-MS/MS	93%	114%	83%	84%
Loperamide	plasma	LC-MS/MS	81%	109%	76%	102%
LSD	plasma	LC-MS/MS	55%	67%	82%	90%
Maprotiline	plasma	LC-MS/MS	56%	68%	82%	80%
MDMA (Extasy)	plasma	LC-MS/MS	89%	121%	74%	95%
Methadone	plasma	LC-MS/MS	43%	83%	52%	87%
Methaqualone	plasma	LC-MS/MS	37%	48%	80%	94%
Metoclopramide	plasma	LC-MS/MS	99%	104%	96%	90%
Metoprolol	plasma	LC-MS/MS	84%	106%	79%	94%
Mianserine	plasma	LC-MS/MS	111%	128%	89%	98%
Mirtazapine	plasma	LC-MS/MS	80%	102%	79%	99%
Moclobémide	plasma	LC-MS/MS	85%	96%	89%	90%
6-mono-acetyl-morphine	plasma	LC-MS/MS	103%	102%	103%	89%
Morphine	plasma	LC-MS/MS	15%	33%	45%	23%
N-Desmethyl-Citalopram	plasma	LC-MS/MS	86%	114%	78%	97%
N-Desmethyl-Clomipramine	plasma	LC-MS/MS	68%	89%	76%	98%
N-Desmethyl-Clozapine	plasma	LC-MS/MS	100%	121%	82%	93%
N-Desmethyl-Mirtazapine	plasma	LC-MS/MS	67%	96%	71%	78%
Nefazodone	plasma	LC-MS/MS	70%	92%	76%	86%
Nicotine	plasma	LC-MS/MS	8%	25%	33%	2%
Nitrazepam	plasma	LC-MS/MS	62%	73%	86%	89%
Nordiazepam	plasma	LC-MS/MS	55%	63%	87%	99%
Norfluoxétine	plasma	LC-MS/MS	34%	57%	59%	91%
Nortriptyline	plasma	LC-MS/MS	29%	33%	89%	90%
Noscapine	plasma	LC-MS/MS	96%	115%	84%	90%
O-desmethyl-Venlafaxine	plasma	LC-MS/MS	95%	96%	101%	99%
Olanzapine	plasma	LC-MS/MS	106%	110%	100%	88%

Opipramol	plasma	LC-MS/MS	113%	138%	82%	83%
Orphenadrine	plasma	LC-MS/MS	71%	86%	82%	104%
Oxazepam	plasma	LC-MS/MS	58%	82%	72%	81%
Papaverine	plasma	LC-MS/MS	101%	123%	83%	94%
Paroxetine	plasma	LC-MS/MS	79%	102%	79%	90%
Pentazocine	plasma	LC-MS/MS	95%	98%	98%	85%
Propanolol	plasma	LC-MS/MS	83%	92%	91%	86%
Propofol	plasma	LC-MS/MS	44%	62%	71%	82%
Protriptyline	plasma	LC-MS/MS	49%	74%	67%	103%
Quinine	plasma	LC-MS/MS	32%	37%	88%	118%
Quetiapine	plasma	LC-MS/MS	93%	107%	88%	105%
Reboxetine	plasma	LC-MS/MS	97%	108%	90%	87%
Risperidone	plasma	LC-MS/MS	88%	96%	92%	90%
Sertindole	plasma	LC-MS/MS	99%	122%	82%	103%
Sertraline	plasma	LC-MS/MS	49%	73%	68%	87%
Sotalol	plasma	LC-MS/MS	17%	98%	18%	60%
Tamoxifène	plasma	LC-MS/MS	58%	102%	57%	82%
Thioridazine	plasma	LC-MS/MS	95%	91%	116%	86%
Tramadol	plasma	LC-MS/MS	89%	98%	93%	96%
Triazolam	plasma	LC-MS/MS	78%	79%	99%	99%
Trimipramine	plasma	LC-MS/MS	32%	48%	68%	86%
Venlafaxine	plasma	LC-MS/MS	98%	119%	83%	105%
Verapamil	plasma	LC-MS/MS	63%	75%	85%	97%
Zolpidem	plasma	LC-MS/MS	106%	109%	97%	101%
Zopiclone	plasma	LC-MS/MS	80%	102%	81%	91%
Acetazolamide	urine	UPLC-MS	65%	57%	115%	108%
Amfepramone	urine	UPLC-MS	87%	95%	92%	90%
Amfetaminil	urine	UPLC-MS	47%	83%	57%	33%
Amiloride	urine	UPLC-MS	62%	59%	106%	83%
Anastrozole	urine	UPLC-MS	32%	102%	31%	1%
Anastrozole	urine	UPLC-MS	82%	102%	80%	98%
Atenolol	urine	UPLC-MS	62%	96%	65%	87%
Bendrofluméthiazide	urine	UPLC-MS	99%	153%	64%	90%
Benzoylecgonine	urine	UPLC-MS	111%	100%	111%	87%
Bromantan	urine	UPLC-MS	100%	349%	29%	95%
Buprenorphine	urine	UPLC-MS	105%	145%	72%	47%
Bupropion	urine	UPLC-MS	120%	108%	111%	114%
Caffeine	urine	UPLC-MS	77%	124%	63%	100%
Cathine	urine	UPLC-MS	89%	179%	50%	80%
Celiprolol	urine	UPLC-MS	119%	99%	120%	101%
Chlorothiazide	urine	UPLC-MS	46%	71%	64%	59%
Chlorphentermine	urine	UPLC-MS	91%	106%	85%	119%
Chlorthalidone	urine	UPLC-MS	43%	41%	105%	92%
Clopamide	urine	UPLC-MS	81%	74%	110%	74%
Crothetamide	urine	UPLC-MS	47%	218%	22%	115%
Dextromoramide	urine	UPLC-MS	97%	121%	80%	85%
Dichlorphenamide	urine	UPLC-MS	57%	48%	118%	87%
Dimetamphetamine	urine	UPLC-MS	94%	108%	87%	101%
Ephedrine	urine	UPLC-MS	78%	72%	108%	73%
Etafedrine	urine	UPLC-MS	63%	125%	50%	76%
Ethacrynic acid	urine	UPLC-MS	68%	65%	105%	69%
Ethylamphetamine	urine	UPLC-MS	81%	156%	52%	71%
Etilefrine	urine	UPLC-MS	88%	77%	115%	108%
Fenfluramine	urine	UPLC-MS	113%	160%	71%	113%
Fenproporex	urine	UPLC-MS	65%	121%	54%	83%
Fentanyl	urine	UPLC-MS	105%	105%	100%	109%
Hydrochlorothiazide	urine	UPLC-MS	20%	31%	63%	66%
Indapamide	urine	UPLC-MS	77%	78%	99%	108%
Isometheptene	urine	UPLC-MS	72%	157%	46%	83%
MDA	urine	UPLC-MS	56%	105%	54%	72%
MDMA	urine	UPLC-MS	97%	153%	63%	107%

Mefenorex	urine	UPLC-MS	104%	183%	57%	104%
Métamphetamine	urine	UPLC-MS	71%	93%	76%	67%
Méthadone	urine	UPLC-MS	61%	57%	108%	84%
Methylamphetamine	urine	UPLC-MS	81%	89%	91%	78%
Methylecgonine	urine	UPLC-MS	6%	83%	8%	15%
Méthylphénidate	urine	UPLC-MS	94%	166%	57%	101%
Metipranolol	urine	UPLC-MS	100%	114%	87%	114%
Metolazone	urine	UPLC-MS	46%	229%	20%	87%
Metoprolol	urine	UPLC-MS	101%	86%	117%	97%
Nadolol	urine	UPLC-MS	100%	92%	109%	106%
Nikéthamide	urine	UPLC-MS	62%	75%	83%	76%
Norbuprénorphine	urine	UPLC-MS	37%	70%	53%	46%
Norfentanyl	urine	UPLC-MS	105%	219%	48%	119%
Oxilofrine	urine	UPLC-MS	81%	97%	84%	102%
Pemoline	urine	UPLC-MS	81%	100%	81%	64%
Pentetrazol	urine	UPLC-MS	31%	47%	66%	75%
Phendimetrazine	urine	UPLC-MS	81%	95%	85%	90%
Phenpromethamine	urine	UPLC-MS	93%	204%	46%	110%
Phentermine	urine	UPLC-MS	88%	213%	41%	96%
Phénylpropanolamine	urine	UPLC-MS	85%	105%	81%	110%
Pholedrine	urine	UPLC-MS	78%	86%	91%	95%
Probenecide	urine	UPLC-MS	77%	450%	17%	93%
Prolintane	urine	UPLC-MS	105%	183%	57%	85%
Propylhexedrine	urine	UPLC-MS	77%	152%	51%	79%
Pseudoephedrine	urine	UPLC-MS	80%	75%	107%	89%
Ritalinic acid	urine	UPLC-MS	48%	100%	49%	90%
RSR 13	urine	UPLC-MS	85%	124%	69%	73%
Salmeterol	urine	UPLC-MS	94%	99%	95%	78%
Sibutramine	urine	UPLC-MS	109%	382%	28%	87%
Strychnine	urine	UPLC-MS	55%	79%	70%	81%
Xipamide	urine	UPLC-MS	94%	115%	82%	91%
Acebutolol	urine	LC-MS	59%	65%	90%	93%
Acetazolamide	urine	LC-MS	26%	28%	92%	31%
Adrafinil	urine	LC-MS	54%	55%	98%	98%
Atenolol	urine	LC-MS	48%	53%	91%	86%
Bendroflumethiazide	urine	LC-MS	69%	72%	96%	106%
Benzoylecgonine	urine	LC-MS	46%	49%	91%	92%
Bethamethasone	urine	LC-MS	77%	72%	106%	105%
Bumetanide	urine	LC-MS	82%	86%	95%	84%
Canrenone	urine	LC-MS	69%	71%	99%	89%
Carteolol	urine	LC-MS	64%	64%	100%	93%
Celiprolol	urine	LC-MS	71%	79%	90%	98%
Chlorothiazide	urine	LC-MS	24%	26%	91%	91%
Chlortalidone	urine	LC-MS	75%	65%	114%	86%
Clopamide	urine	LC-MS	35%	38%	91%	93%
Dexamethasone	urine	LC-MS	79%	80%	98%	109%
Dichlorphenamide	urine	LC-MS	86%	78%	109%	107%
Esmolol	urine	LC-MS	10%	9%	109%	95%
Ethacrinic acid	urine	LC-MS	52%	56%	93%	96%
Fentanyl	urine	LC-MS	91%	97%	94%	91%
Finasteride	urine	LC-MS	73%	75%	94%	95%
Furosemide	urine	LC-MS	83%	98%	85%	84%
Gestrinone	urine	LC-MS	74%	74%	100%	99%
Hydrochlorothiazide	urine	LC-MS	62%	55%	113%	106%
Indapamide	urine	LC-MS	97%	94%	98%	103%
Methylphenidate	urine	LC-MS	78%	95%	83%	92%
Metipranolol	urine	LC-MS	56%	61%	93%	99%
Metolazone	urine	LC-MS	33%	26%	111%	98%
Metoprolol	urine	LC-MS	27%	30%	89%	94%
Modafinil	urine	LC-MS	96%	83%	107%	100%
Nadolol	urine	LC-MS	54%	53%	97%	94%

Piretanide	urine	LC-MS	51%	46%	93%	108%
Probenecide	urine	LC-MS	93%	100%	93%	102%
Sotalol	urine	LC-MS	58%	51%	109%	97%
Strychnine	urine	LC-MS	36%	39%	94%	96%
Torasemide	urine	LC-MS	57%	66%	85%	87%
Xipamide	urine	LC-MS	68%	67%	101%	109%

L'attribution de l'effet matrice a été réalisée avec succès pour chaque composé. Dans certaines situations, le PE, le ME, le RE ou le EY étaient proches mais au-delà de la limite fixée, engendrant des cas inclassables. Dans ces situations, le cas de l'effet matrice le plus proche a été attribué.

De manière générale, aucune influence de la matrice n'a été observée dans 17% des cas (Figure 20a).

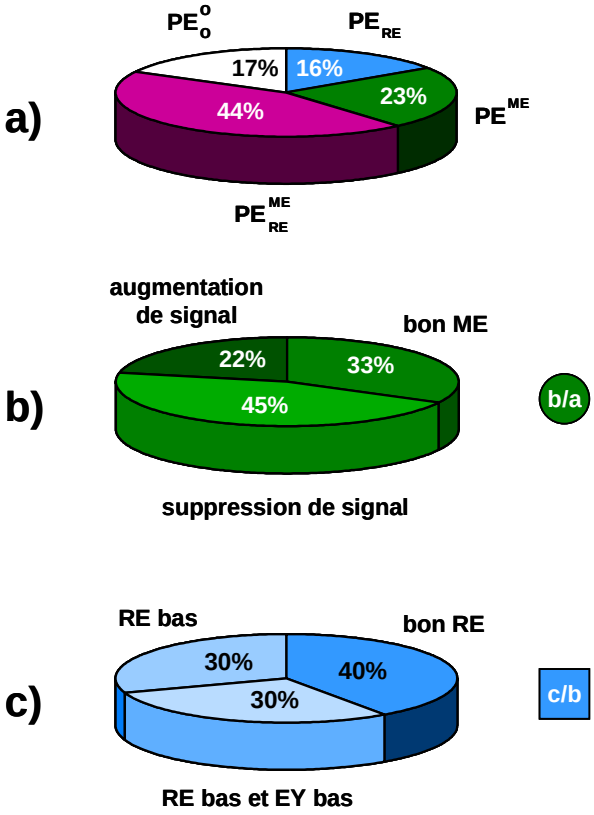


Figure 20 : a) distribution relative des ME et RE sur la totalité des données. PE^{0}_{0} indique les cas avec de bons ME et RE, PE^{ME} fait référence aux cas présentant un ME bas ou haut, PE_{RE} correspond aux cas avec un RE bas et PE^{ME}_{RE} relate les cas avec un ME bas ou haut et un RE bas b) distribution relative des ME bas, bons et hauts c) distribution relative des RE bas et bons.

39% des analytes n'ont été influencés que par un seul paramètre, 16% dû à des problèmes d'extraction et le reste (23%) dû à des altérations du signal MS. La moitié des composés environ (44%) a souffert de la présence de matériel endogène lors des deux étapes du processus analytique. Concernant le ME, 67% des analytes ont subi une altération du signal parmi lesquels 45% étaient

dus à une suppression de signal et 22% à une augmentation de celui-ci (Figure 20b). Enfin, la distribution du RE était équilibrée puisque 60% des composés a présenté une RE bas équitablement partagé entre un RE bas pur (30%) et un RE bas partiellement dû à un EY bas (30%). Les 40% restant n'ont pas subi de problème significatif à l'extraction.

4.5 Actions correctives

Les effets matrices ne doivent pas être éliminés dans tous les cas. En revanche, leur identification et quantification sont, elles, nécessaires. Le rapport de la conférence de Washington en 2007 [115] fixe une limite de variabilité à 15% de la réponse de l'analyte dans six lots indépendants de matrice. Au-delà de cette limite, des mesures correctives doivent être entreprises sur les étapes du processus analytique concernées pour diminuer ou éliminer les effets matrice rencontrés. Un IS deutéré peut être utilisé pour compenser les variations de réponse en LC-MS. Les conditions chromatographiques peuvent également être améliorées de manière à sortir l'analyte de la fenêtre de l'effet matrice (voire chapitre 2). La ré-optimisation de la préparation d'échantillon est également envisageable pour limiter la quantité de composés endogènes. Finalement, l'utilisation d'une autre source d'ionisation moins sujette aux effets matrices, telle que l'APCI et l'APPI, est également une solution d'intérêt puisqu'elle permet de considérablement réduire les effets matrice en LC-MS de manière relativement simple.

4.6 Conclusion

Une classification des effets matrice rencontrés lorsqu'une préparation d'échantillon est réalisée préalablement à l'analyse a été proposée. Huit combinaisons ont été distinguées. Sur la base de cette classification, une arborescence a été élaborée pour l'attribution de la typologie de l'effet matrice rencontré. La préparation de seuls quatre échantillons est requise, permettant le calcul du PE, ME, RE et EY.de manière à illustrer le modèle proposé, un total de 199 composés a été évalué en regard des effets matrice générés avec l'utilisation de trois préparations d'échantillons et trois appareillages LC-MS différents. Chaque analyte a pu être classé parmi l'un des huit cas d'effet matrice identifié. La majeure partie des composés (83%) a subi un effet matrice durant l'une et/ou l'autre des étapes du processus analytique.

5. VALIDATION D'UNE METHODE LC-APPI-MS POUR L'ANALYSE DE L'ALPRAZOLAM ET DU FLUNITRAZEPAM DANS LE SANG HEMOLYSE

5.1 Introduction

Dans ce chapitre, l'aspect quantitatif d'une méthode LC-APPI-MS est étudié *via* le développement et la validation d'une méthode pour l'analyse du flunitrazepam (FLZ), de l'alprazolam (ALZ) et de leurs principaux métabolites, l'α-hydroxyalprazolam (HALZ) et le 7-aminoflunitrazepam (AFLZ) dans le sang hémolysé.

Les benzodiazépines étant aujourd'hui très largement utilisées, leur analyse dans les fluides biologiques est indispensable tant dans le domaine médical que dans le cadre médico-légal. Bon nombre de publications sont déjà parues sur le développement et la validation de méthodes pour leur analyse dans ces matrices en LC-ESI-MS [116;117] et LC-APCI-MS [118;119], mais aucune étude n'a pour l'instant été menée sur les gains de l'APPI pour le couplage de la LC à la MS dans ce domaine.
Cette étude démontre que l'APPI peut non seulement être utilisée pour l'ionisation de composés apolaires mais également dans le cas de l'analyse de molécules assez polaires dans le sang hémolysé, tout en maintenant une très bonne sensibilité et en réduisant les effets matrice couramment rencontrés en ESI et à moindre échelle en APCI.

5.2 Critères de validation

Selon la norme ICH (International Conference on Harmonization), le but d'une procédure de validation d'une méthode analytique est de démontrer qu'elle correspond à l'usage pour lequel elle est prévue en donnant les garanties nécessaires au laboratoire ainsi qu'aux autorités quant à la qualité des résultats obtenus. L'objectif de la méthode analytique est la quantification la plus exacte et la plus précise possible de la quantité d'analyte dans un échantillon inconnu.
Les critères de validation sélectionnés pour cette étude sont la *sélectivité*, la *spécificité*, la *fonction réponse*, la *justesse*, la *fidélité*, l'*exactitude*, la *linéarité* et la *limite de quantification* (LOQ).

5.3 Développement de méthode

Les BZD possédant de larges fenêtres thérapeutiques et toxiques, la méthode d'analyse développée doit permettre la quantification des composés dans une large gamme de concentrations (par exemple, la limite thérapeutique inférieure de l'ALZ est de 0.005 $\mu g \cdot mL^{-1}$ et sa limite toxique supérieure se situe à 0.4 $\mu g \cdot mL^{-1}$ [120]).

5.3.1 SPE

L'ALZ et le FLZ étant des bases faibles relativement hydrophobes, un support hydrophile-lipophile (Oasis HLB) a donc été sélectionné pour cette étude. Chaque étape de l'extraction (chargement, lavage et élution) a été optimisée sur standards aqueux en termes de pH, volume et nature du solvant.

Les échantillons sanguins hémolysés ont été centrifugés et le surnageant simplement dilué avec de l'eau dans un rapport 2 :1. La présence d'éventuels effets matrice a été contrôlée en infusion post-colonne. Puisque aucune altération de signal n'a été observée, la dilution du surnageant a été retenue. L'extraction a été répétée trois fois et les recouvrements sur les élutions calculés pour chacun des composés. Tous étaient compris entre 83% et 119% avec des CV inférieurs à 10%, confirmant l'absence d'effet matrice observée lors de l'infusion post-colonne.

5.3.2 LC-APPI-MS

Malgré l'utilisation de la MS, une séparation chromatographique de résolution suffisante est nécessaire puisque deux couples de composés isobares (FLZ / ALZ-D_5, *m/z* 314 et LRZ / FLZ-D_7, *m/z* 321) sont présents. Ainsi, trois supports chromatographiques ont été comparés (Waters XBridge 100 x 2.1 mm, 3.5 µm, Waters XBridge Shield 100 x 2.1 mm, 3.5 µm et Thermo Hypersil Gold 100 x 2.1 mm, 5 µm). La colonne permettant le meilleur compromis en termes de temps d'analyse et de résolution chromatographique s'est avérée être la XBridge Shield avec une phase mobile composée de tampon acétate 20 mM à pH 5 / ACN 67:33 délivrée en mode isocratique à 200 $\mu L \cdot min^{-1}$ (voir article I). Dans ces conditions, une séparation complète (Rs > 1.5) des composés isobares en moins de 10 minutes a été possible.

De plus, l'influence du solvant d'injection a été évaluée, l'élution des composés de l'Oasis HLB requérant du MeOH pur. Aucune étape d'évaporation/reconstitution n'a été nécessaire puisque les performances chromatographiques ne sont pas altérées par le MeOH pur (Figure 21), probablement grâce au faible volume d'injection (5 µL) et aux facteurs de rétention compris entre 2 et 8.

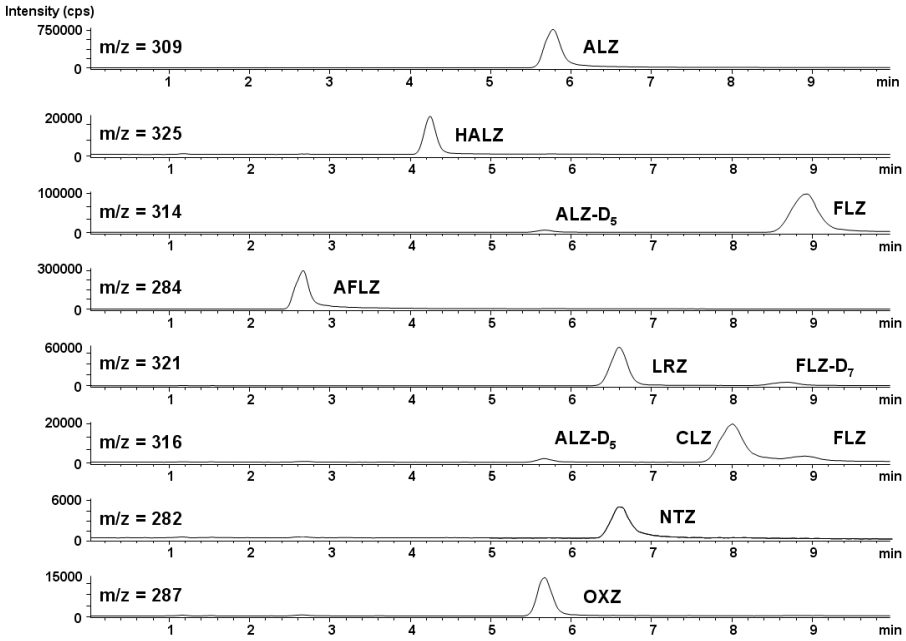


Figure 21 : Chromatogramme d'un standard contenant les BZD testées à 250 ng·mL^{-1} dans MeOH.

D'autre part, le rendement d'ionisation a été comparé en présence d'acétone, de toluène et sans dopant. Les meilleurs résultats ont été obtenus avec l'acétone ajoutée en infusion post-colonne à 10% du débit de phase mobile. Ces conditions ont donc été maintenues pour la suite de l'étude.

5.4 Performances quantitatives de la méthode

L'étude quantitative a été menée sur deux des analytes du mélange et leurs métabolites, à savoir l'ALZ, l'HALZ, le FLZ et l'AFLZ. Des standards internes deutérés (ALZ-D_5 et FLZ-D_7) ont été choisis de manière à compenser les variations de la méthode (préparation d'échantillon, détection, *etc.*) et les éventuels effets matrice.

5.4.1 Sélectivité

La sélectivité d'une méthode d'analyse indique sa capacité à distinguer les composés d'intérêt des autres constituants de l'échantillon (métabolites, matériel endogène, co-médication, *etc.*). Comme mentionné au § 5.3.1, aucun effet matrice n'a été observé. La sélectivité de la méthode a également été évaluée en regard de la matrice par la comparaison des chromatogrammes de sang hémolysé blanc (CAL 00), de sang hémolysé blanc contenant les IS (CAL 0) et d'un standard d'étalonnage à la LOQ (CAL LOQ). Comme illustré sur la Figure 22, aucune interférence n'a été observée au temps de rétention des analytes et de leurs IS, malgré l'utilisation de six sources indépendantes de sang hémolysé.

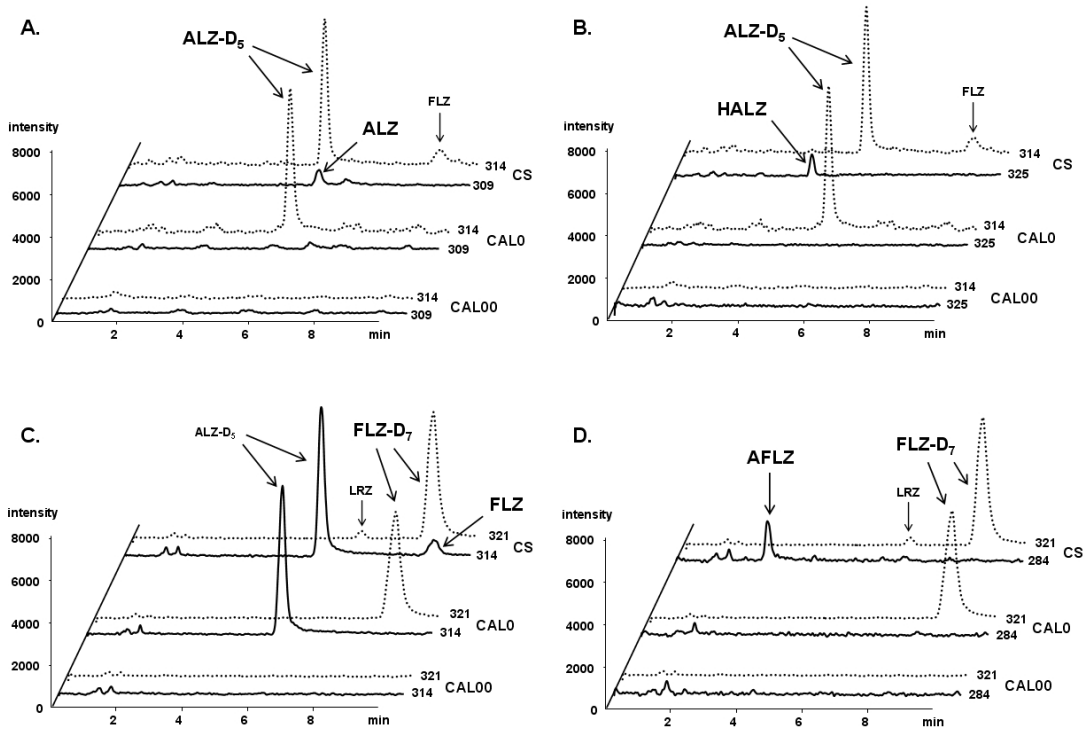


Figure 22 : évaluation de la sélectivité de la méthode pour les quatre composés validés.

5.4.2 Procédure de validation

Les performances quantitatives ont été évaluées sur trois séries (j=3) en accord avec le protocole V5 modifié de la Société Française des Science et Techniques Pharmaceutiques (SFSTP) [121]. Cette version recommande trois niveaux de concentration (k=3) avec deux répétitions (n=2) pour les standards d'étalonnage (CAL) et quatre niveaux de concentration (k=4) avec quatre répétitions (n=4) pour les standards de validation (QC), tous préparés dans la matrice.

- 1 x SST
- 1 x CAL 00
- 1 x CAL 0
- 2 x CAL LOQ
- 2 x CAL 50%
- 2 x CAL 100%
- 4 x QC LOQ
- 4 x QC 4 LOQ
- 4 x QC 50%
- 4 x QC 100%

SST : system suitability test, échantillon reconstitué dans la matrice contenant l'alprazolam (ALZ), l'α-hydroxyalprazolam (HALZ), l'alprazolam-D_5 (ALZ-D_5), le flunitrazepam (FLZ), le 7-aminoflunitrazepam (AFLZ), le flunitrazepam-D_7 (FLZ-D_7), le lorazepam (LRZ), le clonazepam (CLZ), le nitrazepam (NTZ) et l'oxazepam (OXZ).

CAL 00 : échantillon de matrice blanche.

CAL 0 : échantillon de matrice blanche dopée avec ALZ-D_5 et FLZ-D_7.

CAL : standard d'étalonnage, échantillon reconstitué dans la matrice contenant ALZ, HALZ, ALZ-D_5, FLZ, AFLZ et FLZ-D_7 permettant d'établir la droite d'étalonnage.

QC : standard de validation, échantillon reconstitué dans la matrice contenant ALZ, HALZ, ALZ-D_5, FLZ, AFLZ et FLZ-D_7 permettant d'évaluer la justesse de la méthode.

Après la sélection de la meilleure fonction réponse, la justesse (exprimée en biais relatif) et la fidélité (exprimée en coefficient de variation (CV)) ont été déterminées pour chaque niveau de concentration. Les variances de répétabilité et de fidélité intermédiaire ont été calculées sur les concentrations recalculées. Les recommandations SFSTP 1997 [122] ont été suivies pour le calcul de l'intervalle de confiance avec un nombre fixe de degrés de liberté (ddl = j·(n-1)) et un niveau unilatéral de significativité de α=10%. Les intervalles de confiance (erreur totale de la méthode) ont servi à la construction des profils d'exactitude de chaque composé.

5.4.3 Fonction réponse

La fonction réponse exprime, dans l'intervalle de dosage, la nature de la relation liant la réponse analytique mesurée avec la concentration de l'analyte dans l'échantillon. La fonction réponse linéaire est couramment observée, mais d'autres relations sont rencontrées dans la pratique suivant le type de détection utilisé et l'étendue de l'intervalle de dosage. Les modèles de régression évalués dans ce travail sont : fonction linéaire, fonction linéaire forcée par l'origine, fonction linéaire à un point forcée par l'origine (étalon externe), fonction linéaire pondérée (1/x et $1/x^2$), fonction linéaire après transformation par racine carrée (sur x et y), fonction linéaire après transformation logarithmique décimale (sur x et y). Le modèle choisi est celui permettant d'atteindre les meilleures performances d'exactitude pour chaque composé sur toute la gamme de concentrations évaluée. La fonction linéaire pondérée par $1/x^2$ a permis d'obtenir les meilleurs résultats pour l'ALZ, l'HALZ et le FLZ alors que le meilleur modèle pour l'AFLZ est la transformation logarithmique décimale.

5.4.4 Linéarité

Souvent confondue avec la linéarité de la fonction-réponse, la linéarité de la méthode rend compte, selon SFSTP 2003, de sa capacité à rendre des résultats directement proportionnels à la quantité de l'analyte dans l'intervalle de concentrations envisagé. Elle est estimée en recalculant les concentrations des QC à partir de la courbe d'étalonnage et, à l'instar de l'exactitude, elle peut être représentée sous forme de profils (Figure 23).

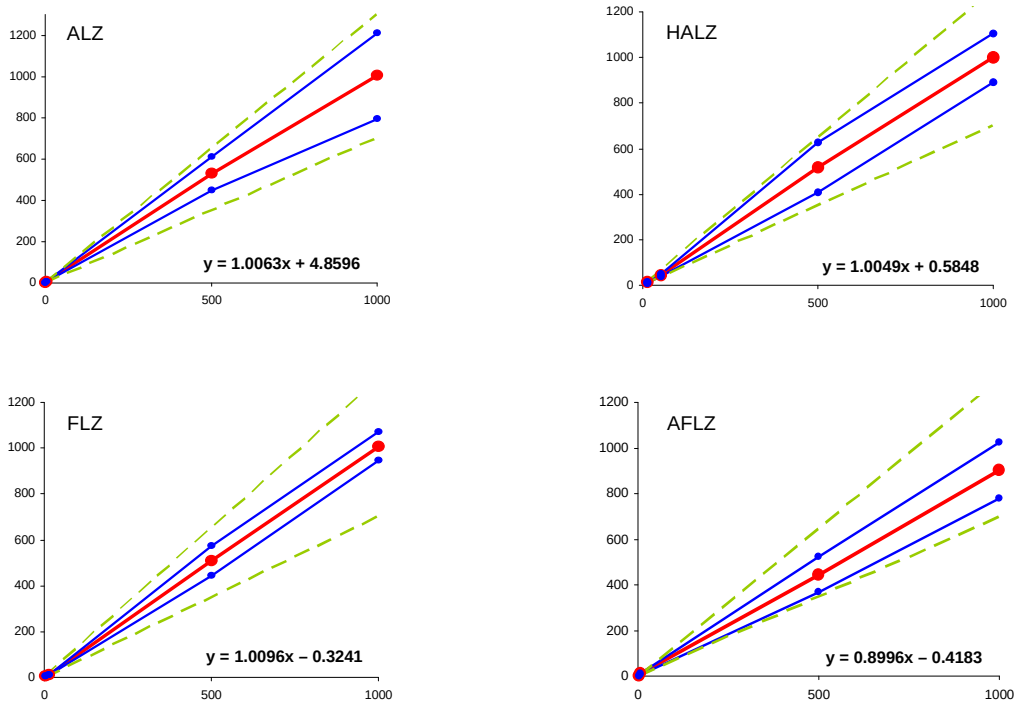


Figure 23 : Profils de linéarité pour l'ALZ, l'HALZ, le FLZ et l'AFLZ.

5.4.5 Limite de quantification

La LOQ définit la plus petite quantité d'analyte mesurable dans l'échantillon, avec une exactitude déterminée. Dans cette étude, la LOQ de chaque composé correspond au niveau de concentration le plus bas puisqu'il a été démontré que l'exactitude de la méthode est acceptable dans tout l'intervalle de dosage pour les quatre composés. La LOQ est donc de 1 $ng \cdot mL^{-1}$, 13 $ng \cdot mL^{-1}$, 3 $ng \cdot mL^{-1}$ et 2 $ng \cdot mL^{-1}$ pour l'ALZ, l'HALZ, le FLZ et l'AFLZ, respectivement.

5.4.6 Justesse

La justesse correspond à l'écart entre la valeur reconnue comme vraie et la concentration moyenne recalculée du standard d'étalonnage. Cette valeur a été exprimée en biais relatif (%) pour chaque niveau de concentration et chaque composé. La justesse était acceptable (limite fixée à ±15%) car inférieure à ±12.8% dans tous les cas à l'exception du FLZ à 12 $ng \cdot mL^{-1}$ (-15.7%) (Tableau 12), toutefois compensée par une bonne fidélité.

Tableau 12 : justesse exprimée en terme de biais relatif pour chaque composé et chaque niveau de concentration.

Justesse	ALZ
Biais relatif (%)	
1 ($ng \cdot mL^{-1}$)	4.2
4 ($ng \cdot mL^{-1}$)	-7.1
500 ($ng \cdot mL^{-1}$)	5.6
1000 ($ng \cdot mL^{-1}$)	0.1

Justesse	HALZ
Biais relatif (%)	
13 ($ng \cdot mL^{-1}$)	2.9
52 ($ng \cdot mL^{-1}$)	-12.8
500 ($ng \cdot mL^{-1}$)	3.6
1000 ($ng \cdot mL^{-1}$)	-0.2

Justesse	FLZ
Biais relatif (%)	
3 ($ng \cdot mL^{-1}$)	4.1
12 ($ng \cdot mL^{-1}$)	-15.7
500 ($ng \cdot mL^{-1}$)	1.4
1000 ($ng \cdot mL^{-1}$)	0.8

Justesse	AFLZ
Biais relatif (%)	
2 ($ng \cdot mL^{-1}$)	2.7
8 ($ng \cdot mL^{-1}$)	3.2
500 ($ng \cdot mL^{-1}$)	-11.0
1000 ($ng \cdot mL^{-1}$)	-9.9

5.4.7 Fidélité

La fidélité rend compte des erreurs aléatoires *via* la dispersion des mesures de plusieurs prises d'essai d'un même échantillon homogène. Elle est exprimée en CV et se décompose en trois niveaux, à savoir la *répétabilité*, la *fidélité intermédiaire* et la *reproductibilité*.

La *répétabilité* correspond à la dispersion des résultats d'essais indépendants obtenus avec la même méthode, sur des individus identiques, dans le même laboratoire, par le même opérateur, sur le même instrument et dans un court intervalle de temps. Il s'agit d'un test intra-laboratoire et intra-jour.

La *fidélité intermédiaire* correspond à la dispersion des résultats d'essais indépendants obtenus avec la même méthode, sur des individus identiques, dans le même laboratoire, par différents opérateurs, sur des instruments différents et dans un intervalle de temps donné. Il s'agit d'un test intra-laboratoire et inter-jour.

Enfin, la *reproductibilité* correspond à la dispersion des résultats d'essais obtenus avec la même méthode, sur des individus identiques, dans divers laboratoires, par différents opérateurs et sur des instruments différents. Il s'agit d'une estimation de la variabilité inter-laboratoire.

La fidélité a été évaluée dans cette étude grâce à une analyse de variances (ANOVA) permettant de déterminer la répétabilité et la fidélité intermédiaire exclusivement. Dans tous les cas, les résultats ont été satisfaisants puisque les CV sont compris entre 3.3% et 10.1% pour la répétabilité et entre 3.3% et 14.3% pour la fidélité intermédiaire (Tableau 13).

Tableau 13 : fidélité exprimée en terme de CV pour chaque composé et chaque niveau de concentration.

Fidélité	ALZ
Répétabilité / Fidélité intermédiaire (CV, %)	
1 (ng·mL^{-1})	9.4 / 13.2
4 (ng·mL^{-1})	4.8 / 12.0
500 (ng·mL^{-1})	8.3 / 8.8
1000 (ng·mL^{-1})	4.5 / 11.2

Fidélité	HALZ
Répétabilité / Fidélité intermédiaire (CV, %)	
13 (ng·mL^{-1})	10.1 / 14.3
52 (ng·mL^{-1})	3.9 / 7.1
500 (ng·mL^{-1})	10.0 / 11.8
1000 (ng·mL^{-1})	4.1 / 5.8

Fidélité	FLZ
Répétabilité / Fidélité intermédiaire (CV, %)	
3 (ng·mL^{-1})	5.8 / 8.2
12 (ng·mL^{-1})	4.4 / 5.9
500 (ng·mL^{-1})	7.1 / 7.1
1000 (ng·mL^{-1})	3.3 / 3.3

Fidélité	AFLZ
Répétabilité / Fidélité intermédiaire (CV, %)	
2 (ng·mL^{-1})	10.0 / 10.4
8 (ng·mL^{-1})	8.1 / 9.8
500 (ng·mL^{-1})	7.4 / 8.7
1000 (ng·mL^{-1})	4.9 / 6.8

5.4.8 Exactitude

L'exactitude correspond à l'erreur totale effectuée sur une mesure, tenant donc compte à la fois de la *justesse* (erreurs systématiques) et de la *fidélité* (erreurs aléatoires). Elle est souvent représentée via un *profil d'exactitude* permettant de visualiser la variabilité totale de la méthode en tenant compte des limites d'acceptation (±30% en milieu biologique). Les profils d'exactitude pour l'ALZ, l'HALZ, le FLZ et l'AFLZ sont représentés sur la Figure 24.

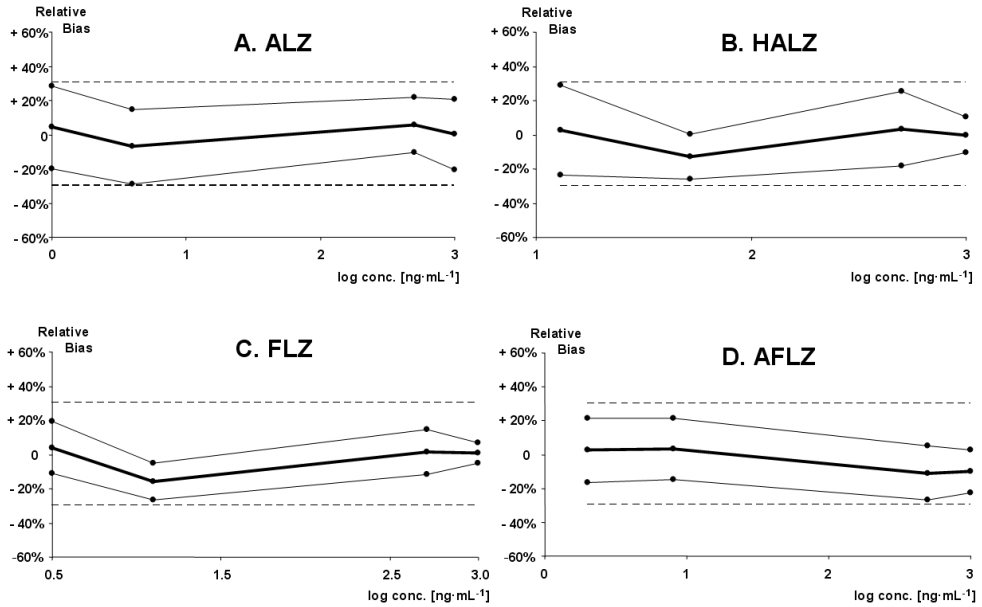


Figure 24 : Profils d'exactitude pour A) l'alprazolam, B) le α-hydroxyalprazolam, C) le flunitrazepam et D) le 7-aminoflunitrazepam.

5.5 Dosage de cas réels

De manière à illustrer l'applicabilité de la méthode à des cas réels, le dosage de l'ALZ, de l'HALZ, du FLZ et de l'AFLZ a été effectué sur deux échantillons réels toxicologiques. Un criblage préalable a permis de déterminer que chacun d'eux ne contenait que l'un des deux principes actifs et le dosage a ensuite été effectué sur les deux échantillons (Figure 25).

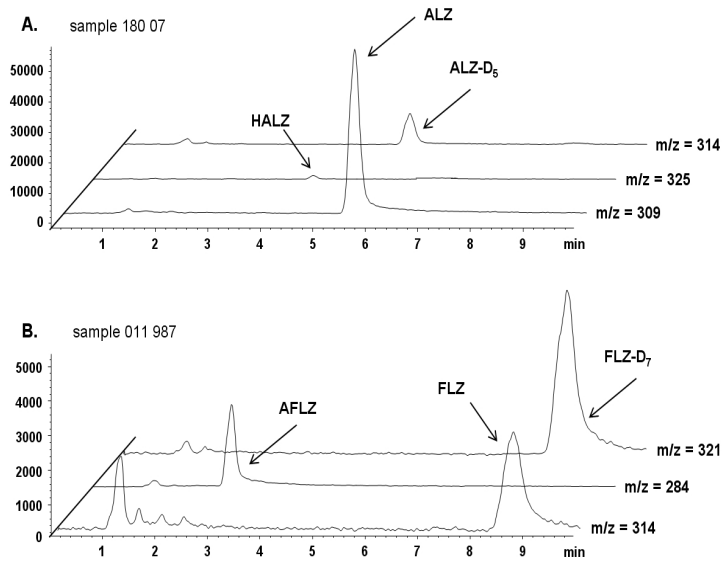


Figure 25 : analyses des cas réels contenant A) de l'aprazolam et B) du flunitrazepam.

La concentration de chaque cas réel a été recalculée sur la base d'une courbe d'étalonnage exécutée le même jour que le dosage et dans les mêmes conditions que lors de la validation (k=3 et n=2). L'analyse du cas réel contenant l'ALZ a été répétée trois fois et celle du FLZ deux fois, en raison d'une quantité d'échantillon insuffisante pour effectuer une troisième mesure. La moyenne de la concentration de chaque échantillon a été calculée selon la formule suivante :

$$\bar{x} = t_{df,\alpha}\sqrt{\frac{s_r^2}{N} + s_g^2}$$

où $\bar{x}$ est la moyenne des concentration obtenues suite à N mesures, $t_{df,\ \alpha}$ est la constante de Student, s^2_r et s^2_g les variance de répétabilité et inter-jour, respectivement.

L'échantillon A contenait 64.6 ng·mL^{-1} ± 18.2 ng·mL^{-1} d'ALZ et 24.7 ng·mL^{-1} ± 3.5 ng·mL^{-1} d'HALZ. L'échantillon B contenait quant à lui 7.6 ng·mL^{-1} ± 0.5 ng·mL^{-1} de FLZ ainsi qu'une concentration de 27.5 ng·mL^{-1} ± 9.6 ng·mL^{-1} d'AFLZ.

5.6 Conclusion

L'utilisation de l'APPI pour le couplage de la LC à la MS présente une excellente alternative aux méthodes habituellement destinées à l'analyse de benzodiazépines dans les matrices biologiques. En effet, l'APPI est compatible avec l'utilisation d'une étape de préparation d'échantillon très simple et rapide par SPE ne nécessitant qu'une simple dilution de l'échantillon préalablement à l'extraction et ne requérant aucune étape d'évaporation / reconstitution avant l'analyse LC-MS. Malgré cette extraction très simple, aucun effet matrice n'a été rencontré et les performance quantitatives étaient satisfaisantes puisque se conformant aux critères d'acceptation (exactitude < ±30%). Finalement, la sensibilité de la méthode est équivalente à celle des méthodes couramment utilisées.

6. REFERENCES

[1] http://www.novartis.com/downloads/newsroom/corporate-press-kit/Research_Drug_Discovery_Process_FR.pdf (accessed on November 14th, 2008)

[2] http://www.novartis.com/downloads/newsroom/corporate-press-kit/Research_Drug_Development_Process_FR.pdf (accessed on November 14th, 2008)

[3] C.Polson, P.Sarkar, B.Incledon, V.Raguvaran, R.Grant, J. Chromatogr. B 785 (2003) 263.

[4] D.Ortelli. Utilisation de la chromatographie liquide couplée à la spectrométrie de masse pour l'analyse de médicaments dans les fluides biologiques. Thèse n° 3296. Université de Genève. 2001.

[5] S.Souverain. Extraction en ligne sur support solide pour l'analyse de composés pharmaceutiques contenus dans des matrices biologiques par chromatographie liquide - spectrométrie de masse. Thèse n°3520. Université de Genève. 2004.

[6] F.Chapuis, V.Pichon, F.Lanza, B.Sellergren, M.C.Hennion, J. Chromatogr. B 804 (2004) 93.

[7] K.Cabrera, D.Lubda, H.M.Eggenweiler, H.Minakuchi, K.Nakanishi, J. High Resolut. Chromatogr. 23 (2000) 93.

[8] H.Minakuchi, K.Nakanishi, N.Soga, N.Ishizuka, N.Tanaka, Anal. Chem. 68 (1996) 3498.

[9] D.B.Robb, T.R.Covey, A.P.Bruins, Anal. Chem. 72 (2000) 3653.

[10] K.A.Hanold, S.M.Fischer, P.H.Cormia, C.E.Miller, J.A.Syage, Anal. Chem. 76 (2004) 2842.

[11] J.A.Syage, K.A.Hanold, T.C.Lynn, J.A.Horner, R.A.Thakur, J. Chromatogr. A 1050 (2004) 137.

[12] M.L.Langhorst, J. Chromatogr. Sci. 19 (1981) 98.

[13] J.N.Driscoll, Am. Lab. 8 (1976) 71.

[14] J.N.Driscoll, F.F.Spaziani, Res. /Dev. 27 (1976) 50.

[15] J.N.Driscoll. J. Chromatogr. 134 (1977) 49.

[16] D.C.Locke, B.S.Dhingra, A.D.Baker, Anal. Chem. 54 (1982) 447.

[17] J.S.M.De Wit, J.W.Jorgenson, J. Chromatogr. 411 (1987) 201.

[18] J.N.Driscoll, D.W.Conron, P.Ferioli, I.S.Krull, K.H.Xie, J. Chromatogr. 302 (1984) 43.

[19] M.A.Baim, R.L.Eatherton, H.H.Hill, Jr., Anal. Chem. 55 (1983) 1761.

[20] H.Borsdorf, E.G.Nazarov, R.A.Miller, Anal. Chim. Acta. 575 (2006) 76.

[21] H.Borsdorf, E.G.Nazarov, R.A.Miller, Talanta. 71 (2007) 1804.

[22] E.G.Nazarov, R.A.Miller, G.A.Eiceman, J.A.Stone, Anal. Chem. 78 (2006) 4553.

[23] G.A.Eiceman, M.E.Fleischer, C.S.Leasure, Int. J. Environ. Anal. Chem. 28 (1987) 279.

[24] G.A.Eiceman, V.J.Vandiver, Anal. Chem. 58 (1986) 2331.

[25] C.S.Leasure, M.E.Fleischer, G.K.Anderson, G.A.Eiceman, Anal. Chem. 58 (1986) 2142.

[26] I.A.Revel'skii, Y.Yashin, V.N.Voznesenskii, V.K.Kurochkin, and R.G.Kostyanovskii. Mass spectrometric analysis of gas mixture. USSR. 83-3546684[1159412]. 24-1-1983. SU.

[27] J.A.Syage, M.D.Evans, K.A.Hanold, Am. Lab. 32 (2000) 24.

[28] E.Marotta, R.Seraglia, F.Fabris, P.Traldi, Int. J. Mass Spectrom. 228 (2003) 841.

[29] D.B.Robb, M.W.Blades, J. Am. Soc. Mass Spectrom. 17 (2006) 130.

[30] L.C.Short, S.S.Cai, J.A.Syage, J. Am. Soc. Mass Spectrom. 18 (2007) 589.

[31] http://webbook.nist.gov/chemistry/ (accessed on November 14th, 2008)
[32] S.T.Tsai, J.C.Jiang, Y.T.Lee, A.H.Kung, S.H.Lin, C.K.Ni, J. Chem. Phys. 111 (1999) 3434.
[33] T.J.Kauppila, R.Kostiainen, A.P.Bruins, Rapid Commun. Mass Spectrom. 18 (2004) 808.
[34] T.J.Kauppila, T.Kuuranne, E.C.Meurer, M.N.Eberlin, T.Kotiaho, R.Kostiainen, Anal. Chem. 74 (2002) 5470.
[35] R.A.Provencal, J.B.Paul, K.Roth, C.Chapo, R.N.Casaes, R.J.Saykally, G.S.Tschumper, H.F.Schaefer, III, J. Chem. Phys. 110 (1999) 4258.
[36] S.Tomoda, K.Kimura, Bull. Chem. Soc. Jpn. 56 (1983) 1768.
[37] E.Marotta, P.Traldi, Rapid Commun. Mass Spectrom. 17 (2003) 2846.
[38] M.Takino, S.Daishima, T.Nakahara, Rapid Commun. Mass Spectrom. 17 (2002) 383.
[39] M.Takino, S.Daishima, N.Taketoshi, J. Chromatogr. A 1001 (2003) 67.
[40] M.Takino, S.Daishima, T.Nakahara, Rapid Commun. Mass Spectrom. 17 (2003) 1965.
[41] M.Takino, K.Yamaguchi, T.Nakahara, J. Agric. Food Chem. 52 (2004) 727.
[42] A.Leinonen, T.Kuuranne, R.Kostiainen, J. Mass Spectrom. 37 (2002) 693.
[43] R.Mol, G.J.de Jong, G.W.Somsen, Electrophoresis. 26 (2005) 146.
[44] S.B.Turnipseed, J.E.Roybal, W.C.Andersen, L.R.Kuck, Anal. Chim. Acta. 529 (2005) 159.
[45] R.Mol, G.J.de Jong, G.W.Somsen, Anal. Chem. 77 (2005) 5277.
[46] A.Delobel, S.Roy, D.Touboul, K.Gaudin, D.P.Germain, A.Baillet, F.Brion, P.Prognon, P.Chaminade, O.Laprevote, J. Mass Spectrom. 41 (2006) 50.
[47] S.Roy, A.Delobel, K.Gaudin, D.Touboul, D.P.Germain, A.Baillet, P.Prognon, O.Laprevote, P.Chaminade, J. Chromatogr. A 1117 (2006) 154.
[48] J.B.Jean-Denis, R.Pezet, R.Tabacchi, J. Chromatogr. A 1112 (2006) 263.
[49] M.Varga, T.Bartok, A.Mesterhazy, J. Chromatogr. A 1103 (2006) 278.
[50] V.Kertesz, G.J.van Berkel, J. Am. Soc. Mass Spectrom. 13 (2002) 109.
[51] R.Mohamed, Y.A.Hammel, M.H.LeBreton, J.C.Tabet, L.Jullien, P.A.Guy, J. Chromatogr. A 1160 (2007) 194.
[52] M.Himmelsbach, M.Haunschmidt, W.Buchberger, C.W.Klampfl, J. Chromatogr. A 1159 (2007) 58.
[53] Y.Hsieh, C.J.G.Duncan, S.Lee, M.Liu, J. Pharm. Biomed. Anal. 44 (2007) 492.
[54] P.Hommerson, A.M.Khan, G.J.de Jong, G.W.Somsen, Electrophoresis. 28 (2007) 1444.
[55] A.Bagag, A.Giuliani, O.Laprevote, Int. J. Mass Spectrom. 264 (2007) 1.
[56] S.S.Cai, K.A.Hanold, J.A.Syage, Anal. Chem. 79 (2007) 2491.
[57] I.Marchi, S.Rudaz, M.Selman, J.L.Veuthey, J. Chromatogr. B 845 (2007) 244.
[58] S.Grosse, T.Letzel, J. Chromatogr. A 1139 (2007) 75.
[59] D.Debois, A.Giuliani, O.Laprevote, J. Mass Spectrom. 41 (2006) 1554.
[60] N.Itoh, Y.Aoyagi, T.Yarita, J. Chromatogr. A 1131 (2006) 285.
[61] L.C.Silva, L.S.O.B.Oliveira, G.D.Mendes, G.Garcia, A.d.S.Pereira, G.De Nucci, J. Chromatogr. B 832 (2006) 302.
[62] Y.Hsieh, K.Merkle, G.Wang, J.-M.Brisson, A.Korfmacher, Anal. Chem. 75 (2003) 3122.
[63] Y.Hsieh, K.Merkle, G.Wang, Rapid Commun. Mass Spectrom. 17 (2003) 1775.

[64] T.J.Kauppila, T.Kotiaho, R.Kostiainen, A.P.Bruins, J. Am. Soc. Mass Spectrom. 15 (2004) 203.
[65] A.Delobel, F.Halgand, B.Laffranchise-Gosse, H.Snijders, O.Laprévote, Anal. Chem. 75 (2003) 5961.
[66] H.Keski-Hynnilä, M.Kurkela, E.Elovaara, L.Antonio, J.Magdalou, L.Luukkanen, J.Taskinen, R.Kostiainen, Anal. Chem. 74 (2002) 3449.
[67] C.Yang, J.Henion, J. Chromatogr. A 970 (2002) 155.
[68] K.S.Hakala, L.Laitinen, A.M.Kaukonen, J.Hirvonen, R.Kostiainen, T.Kotiaho, Anal. Chem. 75 (2003) 5969.
[69] M.J.Greig, B.Bolanos, T.Quenzer, M.R.Bylund Jessica, Rapid Commun. Mass Spectrom. 17 (2003) 2763.
[70] E.R.Trosken, E.Straube, W.K.Lutz, W.Volkel, C.Patten, J. Am. Soc. Mass Spectrom. 15 (2004) 1216.
[71] R.Geyer, A.D.Peacock, D.C.White, C.Lytle, G.J.van Berkel, J. Mass Spectrom. 39 (2004) 922.
[72] H.Moriwaki, M.Ishitake, S.Yoshikawa, H.Miyakoda, J.F.Alary, Anal. Sci. 20 (2004) 375.
[73] G.Wang, Y.Hsieh, W.A.Korfmacher, Anal. Chem. 77 (2005) 541.
[74] E.A.Straube, W.Dekant, W.Voelkel, J. Am. Soc. Mass Spectrom. 15 (2004) 1853.
[75] H.B.Theron, C.Coetzee, F.C.W.Sutherland, J.L.Wiesner, K.J.Swart, J. Chromatogr. B 813 (2004) 331.
[76] S.M.Van Leeuwen, L.Hendriksen, U.Karst, J. Chromatogr. A 1058 (2004) 107.
[77] T.J.Kauppila, P.Oestman, S.Marttila, R.A.Ketola, T.Kotiaho, S.Franssila, R.Kostiainen, Anal. Chem. 76 (2004) 6797.
[78] J.Lembcke, U.Ceglarek, G.M.Fiedler, S.Baumann, A.Leichtle, J.Thiery, J. Lipid Res. 46 (2005) 21.
[79] M.M.Kushnir, R.Neilson, W.L.Roberts, A.L.Rockwood, Clinical biochemistry. 37 (2004) 357.
[80] D.B.Robb, M.W.Blades, J. Am. Soc. Mass Spectrom. 16 (2005) 1275.
[81] Y.Cai, D.Kingery, O.McConnell, A.C.Bach, II, Rapid Commun. Mass Spectrom. 19 (2005) 1717.
[82] T.J.Kauppila, A.P.Bruins, R.Kostiainen, J. Am. Soc. Mass Spectrom. 16 (2005) 1399.
[83] J.L.Gomez-Ariza, A.Arias-Borrego, T.Garcia-Barrera, Rapid Commun. Mass Spectrom. 20 (2006) 1181.
[84] A.K.Kabler, S.Chen, J. Agric. Food Chem. 54 (2006) 6153.
[85] T.J.Kauppila, T.Nikkola, R.A.Ketola, R.Kostiainen, J. Mass Spectrom. 41 (2006) 781.
[86] J.L.Gomez-Ariza, T.Garcia-Barrera, F.Lorenzo, Anal. Chim. Acta. 570 (2006) 101.
[87] A.Yamamoto, N.Kakutani, K.Yamamoto, T.Kamiura, H.Miyakoda, Environ. Sci. Technol. 40 (2006) 4132.
[88] A.Mueller, M.Mickel, R.Geyer, R.Ringseis, K.Eder, H.Steinhart, J. Chromatogr. B 837 (2006) 147.
[89] A.V.Talyzin, Y.O.Tsybin, J.M.Purcell, T.M.Schaub, Y.M.Shulga, D.Noreus, T.Sato, A.Dzwilewski, B.Sundqvist, A.G.Marshall, J. Phys. Chem. A. 110 (2006) 8528.
[90] E.R.Troesken, K.Fischer, W.Voelkel, W.K.Lutz, Toxicology. 219 (2006) 33.
[91] A.Dorcier, P.J.Dyson, J.S.McIndoe, Eur. J. Inorg. Chem. (2003) 4294.
[92] A.Munoz-Garcia, J.Ro, J.C.Brown, J.B.Williams, J. Chromatogr. A 1133 (2006) 58.

[93] M.Papanastasiou, A.W.McMahon, N.S.Allen, A.M.Doyle, B.J.Johnson, K.Keck-Antoine, Polym. Degrad. Stab. 91 (2006) 2675.
[94] J.L.Gomez-Ariza, A.Arias-Borrego, T.Garcia-Barrera, R.Beltran, Talanta. 70 (2006) 859.
[95] L.Song, A.D.Wellman, H.Yao, J.E.Bartmess, J. Am. Soc. Mass Spectrom. 18 (2007) 1789.
[96] J.H.Banoub, B.Benjelloun-Mlayah, F.Ziarelli, N.Joly, M.Delmas, Rapid Commun. Mass Spectrom. 21 (2007) 2867.
[97] J.M.Purcell, R.P.Rodgers, C.L.Hendrickson, A.G.Marshall, J. Am. Soc. Mass Spectrom. 18 (2007) 1265.
[98] Y.S.Ding, D.L.Ashley, C.H.Watson, J. Agric. Food Chem. 55 (2007) 5966.
[99] H.B.Theron, M.J.van der Merwe, K.J.Swart, J.H.van der Westhuizen, Rapid Commun. Mass Spectrom. 21 (2007) 1680.
[100] L.C.Short, K.A.Hanold, S.S.Cai, J.A.Syage, Rapid Commun. Mass Spectrom. 21 (2007) 1561.
[101] L.Song, A.D.Wellman, H.Yao, J.Adcock, Rapid Commun. Mass Spectrom. 21 (2007) 1343.
[102] H.Moriwaki, H.Miyakoda, Rapid Commun. Mass Spectrom. 21 (2007) 1090.
[103] M.Haapala, J.Pol, V.Saarela, V.Arvola, T.Kotiaho, R.A.Ketola, S.Franssila, T.J.Kauppila, R.Kostiainen, Anal. Chem. 79 (2007) 7867.
[104] A.Bacaloni, C.Cavaliere, A.Faberi, P.Foglia, A.Marino, R.Samperi, A.Lagana, Talanta. 72 (2007) 419.
[105] D.B.Robb, M.W.Blades, Anal. Chem. 78 (2006) 8162.
[106] A.Giuliani, D.Debois, O.Laprevote, Eur. J. Mass Spectrom. 12 (2006) 189.
[107] M.Tubaro, E.Marotta, R.Seraglia, P.Traldi, Rapid Commun. Mass Spectrom. 17 (2003) 2423.
[108] J.-P.Rauha, H.Vuorela, R.Kostiainen, J. Mass Spectrom. 36 (2001) 1269.
[109] E.Basso, E.Marotta, R.Seraglia, M.Tubaro, P.Traldi, J. Mass Spectrom. 38 (2003) 1113.
[110] I.Dzidic, D.I.Carroll, R.N.Stillwell, E.C.Horning, J. Am. Chem. Soc. 96 (1974) 5258.
[111] S.Souverain, S.Rudaz, J.L.Veuthey, J. Chromatogr. A 1058 (2004) 61.
[112] C.Schafer, D.Lubda, J. Chromatogr. A 909 (2001) 73.
[113] B.K.Matuszewski, M.L.Constanzer, C.M.Chavez-Eng, Anal. Chem. 75 (2003) 3019.
[114] R.Bonfiglio, R.C.King, T.V.Olah, K.Merkle, Rapid Commun. Mass Spectrom. 13 (1999) 1175.
[115] C.T.Viswanathan, S.Bansal, B.Booth, A.J.DeStefano, M.J.Rose, J.Sailstad, V.P.Shah, J.P.Skelly, P.G.Swann, R.Weiner, Pharm. Res. 24 (2007) 1962
[116] H.Yuan, Z.Mester, H.Lord, J.Pawliszyn, J. Anal. Toxicol. 24 (2000) 718.
[117] M.Cheze, G.Duffort, M.Deveaux, G.Pepin, Forensic Sci. Int. 153 (2005) 3.
[118] A.Bugey, S.Rudaz, C.Staub, J. Chromatogr. B 832 (2006) 249.
[119] K.L.Duffin, T.Wachs, J.D.Henion, Anal. Chem. 64 (1992) 61.
[120] M.Schulz, A.Schmoldt, Pharmazie. 58 (2003) 447.
[121] P.Hubert, J.J.Nguyen-Huu, B.Boulanger, E.Chapuzet, STP Pharma Pratiques. 13 (2003) 101.
[122] E.Chapuzet, N.Mercier, S.Bervoas-Martin, B.Boulanger, STP Pharma Pratiques. 7 (1997) 169.

Chapitre 2 : Articles

Article I

Atmospheric pressure photoionization for coupling liquid-chromatography to mass spectrometry : a review.

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Talanta (*In press, doi:10.1016/j.talanta.2008.11.031)*

ABSTRACT

This review presents the state-of-the-art techniques that couple liquid chromatography and mass spectrometry via atmospheric pressure photoionization (APPI). The different ionization mechanisms are discussed as well as the influence of the mobile phase composition, the nature of the dopant, etc. A comparison with other ionization sources, such as electrospray ionization and atmospheric pressure chemical ionization, is reported, and the combination of APPI with these sources is also discussed. Several applications, covering the time period of 2005 - 2008, for the analysis of drugs, lipids, natural compounds, pesticides, synthetic organics, petroleum derivatives, and other substances are presented.

Keywords : atmospheric pressure photoionization ; APPI ; photoionization ; PI

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Abbreviations list

abbreviation	meaning
AA	anion attachment
API	atmospheric pressure ionization
APCI	atmospheric pressure chemical ionization
APPI	atmospheric pressure photoionization
BGE	background electrolyte
CE	capillary electrophoresis
CI	chemical ionization
CX	charge exchange
CZE	capillary zone electrophoresis
EA	electronic affinity
EC	electron capture
EI	electron ionization
ESI	electrospray ionization
FID	flame ionization detector
IE	ionization energy
LC	liquid chromatography
LOD	limit of detection
LOQ	limit of quantification
MEKC	micellar electrokinetic chromatography
MEEKC	microemulsion electrokinetic chromatography
MS	mass spectrometry
PA	proton affinity
PAH	polycyclic aromatic hydrocarbon
PI	photoionization
PID	photoionization detector
SE	solvation energy
SDS	sodium dodecyl sulfate
S / N	signal to noise
VUV	vacuum-ultraviolet

1. Introduction

Due to its universality, selectivity and sensitivity, mass spectrometry (MS) is considered the gold standard detector for liquid chromatography (LC). The first coupling of LC to MS was performed in the 1970s but became widely used only with the appearance of atmospheric pressure ionization (API) sources in the early 1990s. Among these interfaces, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are currently the most widely employed API sources. ESI is particularly adapted to the analysis of polar molecules, and its ionization occurs in the liquid phase, while APCI allows the ionization of less polar molecules in the gas phase. Atmospheric pressure photoionization (APPI) is the last soft ionization technique able to ionize those molecules that are poorly amenable to ESI and APCI [1-3].

Photoionization (PI) was already used at low pressure as a detection method (PID) in gas chromatography (GC) in the mid-1970s. PID provided better sensitivity for apolar compounds (benzene), than a flame ionization detector (FID), and a dynamic range greater than 10^7 [4-7]. During the same decade, PI was also used as a detection method in LC, and the early studies in LC-PID [8] presented a similar set-up to the one employed in GC-PID. However, only a few applications of these techniques were published [9-11].

The use of PI at atmospheric pressure was first presented by Baim *et al.* in 1983 where it was coupled with ion mobility spectrometry (IMS) [12]. It was implemented with the same vacuum-ultraviolet (VUV) lamp used in GC [1]. Various examples of APPI coupled to IMS were proposed [12-15], and other applications have been recently published [16-18]. Moreover, in 1991, one patent reported the coupling of PI at atmospheric pressure with MS [19]. This study demonstrated the possibility of a direct infusion of analytes operating with helium as a carrier gas. Finally, the first studies using APPI as an ionization source for coupling LC to MS were published in 2000 by Robb *et al.* [1] and Syage *et al.* [20] (Table 1).Thanks to these results, two interfaces are currently commercially available, namely, the PhotoSpray and the PhotoMate sources (see section 2.3).

Table 1 : Historical overview of PI.

subject	1st publication	authors
GC-**PID**	middle 70's	Driscoll *et al.*
LC-**PID**	middle 70's	Schermund *et al.*
APPI-IMS	1983	Baim *et al.*
APPI-MS	1991	Revel'skii *et al.*
LC-**APPI**-MS	2000	Robb *et al.* & Syage *et al.*

The number of publications related to the use of PI has rapidly grown since its implementation at atmospheric pressure. Indeed, the use of PI at reduced pressure yields only poor ionization due to the low sample density in the ionizing region [21].

The objective of this review is to present the state-of-the-art of APPI and its recent developments. Indeed, only applications from papers published since 2005 are considered, as two reviews on APPI concerning the time periods 2000-2002 [22] and 2003-2004 [23] were previously published.

2. Theory

2.1 Reactional mechanisms

PI is based on the interaction of a photon beam produced by a discharge lamp with the vapors formed by the nebulization of a liquid solution. PI occurs in several steps. First, the absorption of a photon ($E = h\nu$) by a molecule (M) leads to an electronically excited molecule :

$$M + h\nu \rightarrow M^* \quad (1)$$

If the ionization energy (IE) of the analyte is lower than $h\nu$, the molecule releases an energetic electron (E_{e^-} (max) = $h\nu$ - IE_M) leading to the corresponding odd-electron cation (a phenomenon typically occurring with molecules with conjugated double bonds, such as aromatic compounds [24]) :

$$M^* \rightarrow M^{+\bullet} + e^- \quad (2)$$

At atmospheric pressure, the ion's free pathway is 65 nm [24]. Thus, molecular ions ($M^{+\bullet}$) with an unpaired electron show a tendency to react in collisional environments [2]. Moreover, molecules with low IE and/or high gas phase basicity, called proton affinity (PA), tend to dominate the positive ion spectra due to their high collision frequency [25].

However, when the IE > $h\nu$, M* may undergo a de-excitation process, such as photodissociation (3), photon emission (4) or collisional quenching (5) with a non-excited molecule (C) :

$$M^* \rightarrow A + B \quad (3)$$

$$M^* \rightarrow M + h\nu \quad (4)$$

$$M^* + C \rightarrow M + C^* \quad (5)$$

In such cases, the use of a preferentially ionized substance, called a dopant (D), has been proposed to promote the ionization of M :

$$D + h\nu \rightarrow D^{+\bullet} \quad (6)$$

The dopant is added in large quantities compared to the analytes, and it acts as an intermediate between the photons and the analytes. The dopant must therefore produce photoions with a high recombination energy and/or a low PA. The ionization mechanism depends on the PA values of the involved molecules (dopant, solvent, analyte) and on their capacity to capture an electron in the gas phase, called electron affinity (EA) [1]. Two mechanisms can occur, namely charge transfer (7) and proton transfer (8) :

$$D^{+\bullet} + M \rightarrow D + M^{+\bullet} \qquad \text{if } EA_D > EA_M \qquad (7)$$

$$D^{+\bullet} + M \rightarrow [D\text{-}H]^{\bullet} + [M+H]^{+} \qquad \text{if } PA_M > PA_{[D\text{-}H]\bullet} \qquad (8)$$

However, a dopant molecule can react only once. The dopant can also be used to improve the ionization yield of the analyte (even if IE < $h\nu$) because photons cannot penetrate deeply into the dense mixture of gases [26] (the photon beam produced by a krypton lamp loses 50% of its intensity each 1.5 mm [18]). Therefore, the probability for direct ionization of analytes, present in small proportion compared to solvent molecules, is very low.

Moreover, in the case of a chromatographic separation, solvent molecules are also present and can react according to equations 9 and 10 :

$$D^{+\bullet} + nS \rightarrow [D\text{-}H]^{\bullet} + [S_n+H]^{+} \qquad \text{if } PA_S > PA_{[D\text{-}H]\bullet} \qquad (9)$$

$$[S_n+H]^{+} + M \rightarrow nS + [M+H]^{+} \qquad \text{if } PA_M > PA_S \qquad (10)$$

It has to be noted that APPI is operated in the gas-phase because the IE values are generally lower in the liquid phase, making it impossible to discriminate between the analyte and solvent molecules (*i.e.*, 6.1eV in the liquid-phase vs. 12.6eV in the gas-phase for water [9]).

2.2 Light sources

The greater part of the studies conducted in APPI were performed with a krypton lamp, which can produce photons of 10.03eV and 10.64eV in a 4:1 ratio [27;28]. Krypton was selected mainly because most analytes have lower IE values than the photon's energy, while commonly used solvents and gases present in the source (O_2, N_2 etc.) have higher values. It should be noted that, in general, the IE tends to decrease with the size of the organic molecules (see anthracene, naphthalene and benzene series, Table 2) [3].

Besides krypton, xenon and argon were also used. Xenon was employed by Locke *et al.* in the early years of PI [11], and it presents the best penetration depth in the ionizing region but is less adapted to efficient photoionization due to the low energy of produced photons. Argon produces more energetic photons than krypton (11.7eV), which are absorbed by the solvent only after a few millimeters. Thus, argon lamps give higher intensities (100 times) in the solvent ions and produce more abundant molecular ions than krypton lamps [28]. Regarding the ionization efficiency of the

analytes as a function of the flow rate, krypton lamps produce a better S/N ratio at a low solvent flow rate, whereas argon lamps are better at higher solvent flow rates.

Table 2 : Gas-phase IE and PA values of commonly used solvents and compounds from [21].

compound	IE (eV)	PA (kJ·mol^{-1})
nitrogen	15.58	493.8 [1]
water	12.62	691.0 [4]
acetonitrile	12.20	779.2 [3]
oxygen	12.07	421.0 [4]
	Ar : 11.7	
chloroform	11.37 [3]	n.a.
methanol	10.84	754.3 [3]
acetic acid	10.65 [4]	783.7 [4]
	Kr : 10.6	
isopropanol	10.17	793.0 [1]
hexane	10.13	n.a.
ammonia	10.07 [4]	853.6 [4]
	Kr : 10.0	
heptane	9.93	n.a.
isooctane	9.80	n.a.
methanol dimer	9.74 [2]	899.1 [2]
acetone	9.70	812.0 [3]
tetrahydrofuran	9.4 [1]	822.1 [1]
pyridine	9.26	930.0 [1]
testosterone	9.2 [3]	880.0 [3]
benzene	9.24	750.4 [3]
furan	8.88	803.4 [1]
toluene	8.83	784.0 [3]
	Xe : 8.4	
anisole	8.2 [3]	839.6 [3]
naphtalene	8.14 [3]	802.9 [3]
anthracene	8.10 [3]	877.3 [3]
acridine	7.8 [3]	972.6 [3]
triethylamine	7.53	981.8 [1]
benzyl radical	7.2 [4]	831.4 [4]

[1] http://webbook.nist.gov/chemistry/
[2] [139]
[3] [24]
[4] [31]
n.a. : not available.

2.3 Commercially available sources

Only two APPI sources are commercially available. The first was based on the prototype developed by Robb *et al.* in 2000 (Figure 1) and is available under the PhotoSpray trademark name.

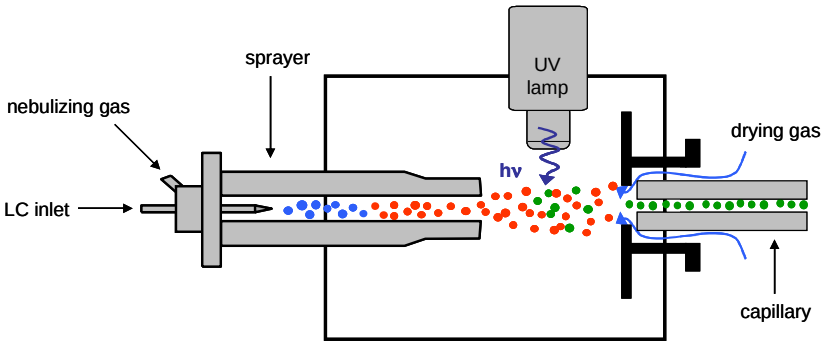

Figure 1 : Schematic representation of the APPI prototype source used by Robb *et al.* [1].

The second source is called PhotoMate and differs by its orthogonal geometry (Figure 2). Both sources incorporate magnesium fluoride (MgF_2) lamp windows to ensure the photon's transmission [1].

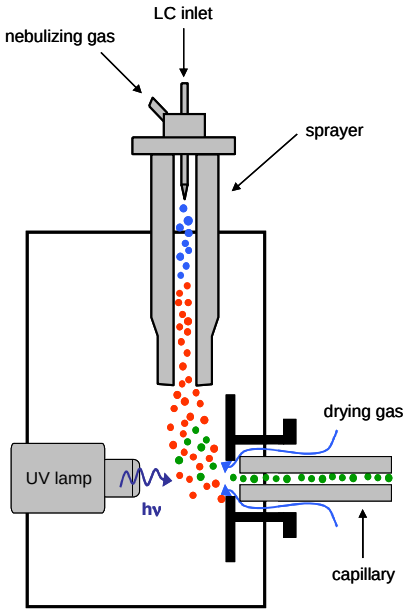

Figure 2 : Schematic representation of the PhotoMate source inspired from Agilent documents.

The main difference between both sources stands in the compulsory presence of a dopant for the PhotoSpray source. With the latter, the offset potential (the electrical potential applied between the lamp and the mounting bracket) has a tremendous effect on the method's sensitivity because the ion production is low (10 nA of ion current measured at the MS curtain plate versus 2.5 µA in APCI) [1]. This phenomenon is not observed on the PhotoMate source since the lamp ignition is different. Finally, the latter was also found to be able, under particular conditions, to ionize highly polar compounds (see section 9).

3. LC solvents in APPI

Solvents commonly used in reversed-phase liquid chromatography (RPLC) have been studied regarding their influence on the ionization process. This section is dedicated to the most commonly used solvents in RPLC, namely, methanol, acetonitrile and water. The influence of buffers will be discussed in section 8.

3.1 Methanol

In spite of its low IE (see Table 2), the use of methanol in aqueous mobile phases provided a 2- or 3-fold increase in the production of molecular ions $[M]^{+\bullet}$ of naphthalene and diphenyl sulfide in comparison to water / acetonitrile mixtures, using the source developed by Robb *et al.* However, almost no influence of solvent's nature was observed on protonated molecules $[M+H]^+$ of carbamazepine and acridine [1]. This observation was recently confirmed with the PhotoMate ionization of PAH, providing much higher sensitivities for the molecular ions in pure methanol than in pure acetonitrile [28].

Furthermore, methanol was found to produce clusters in the gas phase [29;30], which concentration was correlated to the production of protonated analytes. Indeed, the influence of charged methanol clusters on the ionization of PAH was investigated [28]. Production of $[PAH+H]^+$ was maximum in the 300-400 $\mu L \cdot min^{-1}$ range, corresponding to the highest concentration of the protonated methanol dimer. A proton transfer from the protonated methanol dimer to a PAH molecule (due to the PA value of the protonated methanol dimer generally lower than that of PAH) was suggested. This mechanism is similar to the signal enhancement obtained with a dopant [28].

A mathematical model was also proposed for calculating the proportion of methanol clusters in the gas region. It was estimated that, at 50 $\mu L \cdot min^{-1}$, the vapor contained 99.98% of the methanol monomer (IE = 10.84eV) and 0.02% of the dimer (IE = 9.74eV) [28]. When using a krypton lamp, only the dimer could be ionized. This occurred through dissociative ionization :

$$[CH_3OH]_2 + h\nu \rightarrow [CH_3OH]_2^+ + e^- \quad (11)$$

$$[CH_3OH]_2^+ \rightarrow [CH_3OH+H]^+ + CH_3O \text{ (or } CH_2OH) \quad (12)$$

The cluster size was found to depend on the applied cone voltage using the PhotoSpray. At low values (< 10 V), the dimer and the trimer were dominant, whereas, at higher voltages, both clusters were accelerated during their introduction in the low vacuum MS region, leading to their fragmentation into protonated monomer and dimethyl ether ($[CH_3]_2OH^+$) ions [31]. Besides, formation of large protonated clusters was favored by the large binding energy between methanol and a protonated cluster, $CH_3OH + [CH_3OH]_nH^+$ (-33 $kcal \cdot mol^{-1}$ for n=1, -22 $kcal \cdot mol^{-1}$ for n=2 and -16 $kcal \cdot mol^{-1}$ for n=3 [32]). In the presence of water in the mobile phase, clusters such as $[CH_3OH]_n \cdot H_2O$ were also formed, and their occurrence increased as the water/methanol ratio approached one.

Enlargement of such clusters by the addition of a methanol molecule was thermodynamically favored (-24.6 kcal·mol^{-1}) [33].

3.2 Acetonitrile

In comparison with methanol, acetonitrile ionization with the PhotoMate was found to be lower, probably due to its higher IE value (12.2eV) [28]. Robb *et al.* also observed that proton transfer was almost not affected by the solvent's nature, but charge transfer was lowered by a factor of 2-3 in acetonitrile [1]. In order to investigate ionization mechanisms in presence of acetonitrile, Marotta *et al.* observed that the latter participated to the formation of the $[M+H]^+$ ions of furocumarins [21;34]. Indeed, in the presence of CD_3CN, protonated molecules were replaced by $[M+D]^+$ ions, proving unambiguously the role of acetonitrile in the proton transfer mechanism. An irradiated molecule of acetonitrile gave various structures reported in Figure 3a [21;34]. For various reasons, structure 3 appeared to be the most stable isomer. With a calculated IE value of 9.5 eV, structure 3 could be ionized to the corresponding odd-electron ion (Figure 3b), which might then sequentially react with neutral acetonitrile and water. Finally, the charged cluster could ionize the analytes by proton transfer (Figure 3c) [21;34].

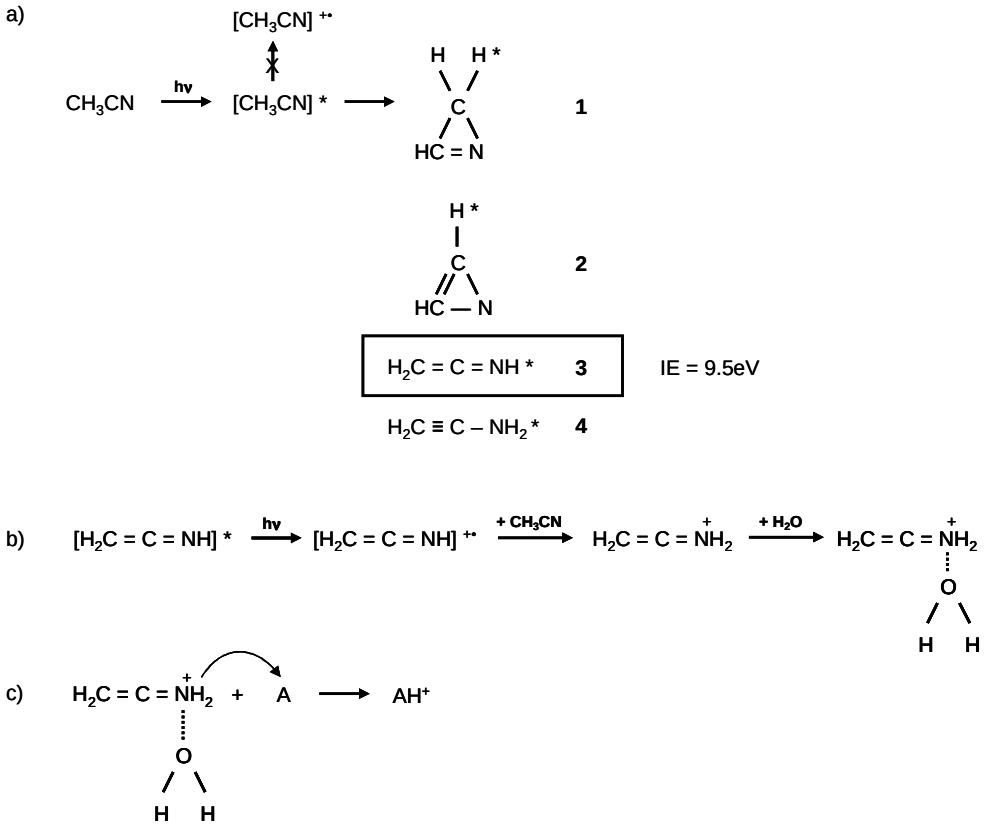

Figure 3 : a) acetonitrile photoisomerization followed by b) photoionization. c) ionization by proton transfer from protonated acetonitrile to an analyte molecule.

However, two consecutive bimolecular reactions with continuous and incoherent lights sources (such as krypton or argon lamps) were deemed unlikely by Short *et al.* [28]. According to their results, the $[M]^{+\bullet}$ ions produced by direct ionization can abstract a hydrogen atom from acetonitrile. This hypothesis relied on the higher production of protonated acetonitrile clusters with a krypton lamp than with an argon lamp. Acetonitrile was also found in the source atmosphere in a variety of forms, mainly the protonated monomer, dimer and propionitrile ions ($[CH_3CH_2CN]H^+$). The cone voltage values seemed to have an influence on the cluster populations. Indeed, the dimer was essentially found at low cone voltages (13), while the propionitrile ion was present at higher values (14).

$$[CH_3CN+H]^+ + CH_3CN \rightarrow [CH_3CN]_2H^+ \quad (13)$$

$$[CH_3CN+H]^+ + CH_3CN \rightarrow [CH_3CH_2CN]H^+ + HCN \quad (14)$$

In presence of water, acetonitrile formed homologous clusters to those observed with water / methanol (see section 3.1). However, ionization of the analytes was higher in pure acetonitrile than in water/acetonitrile mixtures, independently on the lamp (Xe, Kr, and Ar) [28].

3.3 Water

The influence of water on the photoionization was not widely investigated, and no mechanistic studies are available. Nevertheless, Giuliani *et al.* studied the ionization of hexamethonium bromide salt (a bisquaternary salt) in aqueous solutions. It appeared that only minor in-source fragmentations occurred in the APPI conditions, namely, dequaternization (*i.e.*, loss of a single methyl group) and charge separation from the two major precursors (M^{2+} and $[M+Br]^+$). Moreover, fragmentation was greatly enhanced in presence of a dopant (see section 4.2) [35]. Rauha *et al.* investigated the effect of various solvents on the ionization of flavonoids by proton transfer with the PhotoSpray and the highest responses were observed in pure water with toluene as a dopant [36].

As a conclusion, it is recommended to utilize as far as possible methanol rather than acetonitrile when compounds ionized *via* charge transfer are analyzed. However, in the case of analytes ionized by proton transfer, the solvent has only a small influence and both acetonitrile and methanol can be employed. Indeed, proton transfer is often attributable to the presence of protonated clusters of solvent molecules which reactivity (related to their PA) seems to be comparable for both solvents. Water favors proton transfer, probably due to its low PA (691 $kJ \cdot mol^{-1}$, see Table 2), inferior to anthracene (877.3 $kJ \cdot mol^{-1}$) and naphthalene (802.9 $kJ \cdot mol^{-1}$). However no information was available for charge transfer.

4. Dopant-assisted APPI

The use of a dopant was already applied in 1994 with acetone and in 1997 with benzene, toluene and xylene for PI-IMS ionization enhancement. Benzene was also employed in 1991 as a charge transfer media for improving the chemical ionization in APCI [37]. Regarding LC-APPI-MS, acetone [38-61] and toluene [26;27;51;54;55;62-104] have been largely used, studied and compared, whereas anisole [24;35;49;51;55;59;60;77;78], benzene [66;105], hexafluorobenzene [35;59] and THF [53] have been employed to a lesser extent. The use of dopants in the negative mode is presented in section 5.

4.1 Benzene

The use of benzene as dopant mainly leads to the formation of protonated analytes, while the production of molecular ions is less efficient. In order to understand reactional mechanisms involved, Tubaro *et al.* investigated the ionization of benzene and toluene with the PhotoMate source [105]. The formation of benzene $[M]^{+\bullet}$ ions was found to be related to its partial pressure in the source. At low values, only molecular ions of benzene were formed, while, with increasing partial pressures, the $[M]^{+\bullet}$ ions almost disappeared, and six new ions emerged (m/z = 94, 156, 170, 171, 186, 187), implicating oxygen present in the source (Figure 4a and b). The produced phenol radical cation reacted with a neutral benzene molecule leading to the production of ions at m/z = 170 and m/z = 171 (Figure 4c). Analytes ionization was therefore attributed to a proton transfer from ion m/z = 171 and possibly to a charge transfer from its radical cation (m/z = 170). As the phenol radical cation participates to the reactional mechanisms, the addition of large quantities of phenol to benzene was tested. It resulted that the phenol radical cation (m/z = 94) reacted with a neutral phenol molecule to produce ions at m/z = 186 and m/z = 187 (Figure 4d), also able to transfer a charge (m/z = 186) or a proton (m/z = 187). It seems therefore that phenol could also be used as a dopant.

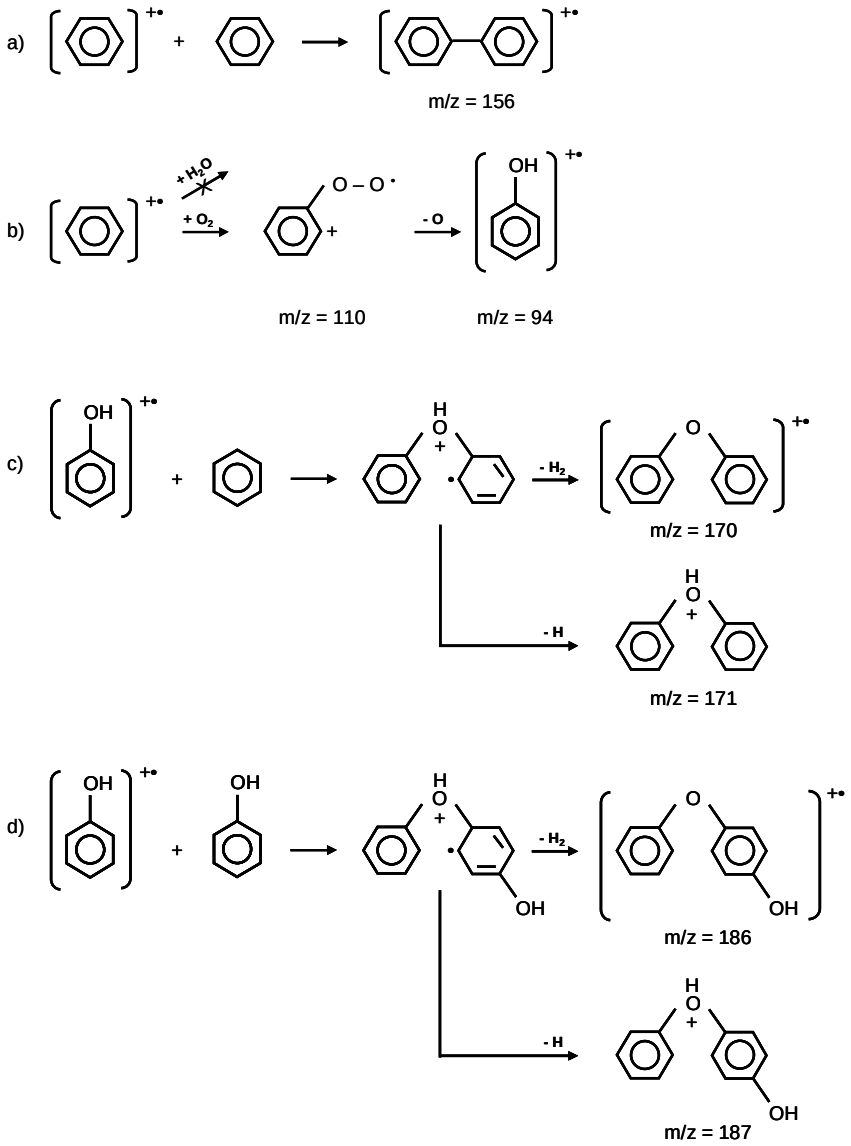

Figure 4 : Chemical reactions leading to the formation of the six ions detected at high partial pressure of benzene. Formation of m/z = 156 (a), m/z = 94 (b), m/z = 186 & 187 (c) when phenol is added in the source and m/z = 170 & 171 (d) with no phenol adjunction.

4.2 Acetone and toluene

In order to explain ionization of analytes assisted with acetone and toluene as dopants, investigations of reactional pathways are divided into two sections. Ionization mechanisms of both dopants are first described in section 4.2.1 whereas section 4.2.2 is dedicated to the reactional pathways between these dopant ions and the analytes.

4.2.1 Dopant-only situation

Robb *et al*. already hypothesized in 2000 that dopant ionization may result in several reactions. The predominant mechanism was identified as the reaction between dopant ions and solvent molecules. The solvent, once charged, ionized in turn the analytes either by proton or charge transfer (depending on the analytes' PA and IE values). This early suggestion was partially confirmed by Tubaro *et al.* [105] regarding the ionization mechanisms of toluene using the orthogonal commercially available source (PhotoMate). Reactions similar to those presented in section 04.1 with benzene were observed with toluene. The intermediate reactive ion (homologous to phenol with benzene) was cresol, already observed in a previous study [31]. Nevertheless, more complex ions were produced at higher m/z values with toluene than with benzene, which involved a large number of reactional pathways, thereby complicating the elucidation of the reaction mechanisms. Furthermore, Robb and Blades showed that the production of the toluene photoions using the PhotoSpray source was directly proportional to the dopant flow and/or the lamp current [27;80]. From 0.2 mA to 2.0 mA, the signal intensity of the toluene ions was even found to increase by 300% [27]. However, the production of toluene ions was inversely proportional to the solvent flow rate, the key factor for dopant ion production being the dopant/solvent ratio.

4.2.2 Analytes ionization

In the first study dedicated to LC-APPI-MS with the prototype linear source, Robb *et al*. observed the influence of the dopant nature on the ionization of high PA (carbamazepine and acridine) and low PA (naphthalene and diphenyl sulfide) compounds [1]. With toluene, proton transfer leading to $[M+H]^+$ ions was the most efficient ionization mechanism [24;31]. Analytes with high PA presented signals about 100 times higher than those obtained from the low PA molecules. Acetone could ionize analytes only through proton transfer and was less efficient than toluene, regardless the source [1;2].

Ionization of naphthalenes with toluene was also studied by Kauppila *et al.* with the PhotoSpray [31]. In presence of water, hexane or chloroform, toluene was ionized only in $[C_7H_8]^{+\bullet}$ because PA values of the solvents are sufficiently low (*i.e.*, no proton transfer possible from the dopant to the solvent). Therefore, abundant ionization of the analytes occurred by charge transfer only with these solvents. Nevertheless, with acetonitrile or methanol, clusters of both solvents possessing higher PA values than toluene were found to be protonated by $[C_7H_8]^{+\bullet}$, leading to their disappearance. As reported in section 3.2, methanol formed monomers, dimers and trimers also in presence of toluene. The monomer formation was favored by increasing the vaporizer temperature from 200℃ to 500℃, indicating that the latter had an effect on the APPI process. Besides, it was shown that both proton and charge transfer were not the unique ionization pathways with toluene. Indeed, in the presence of chloroform as solvent, $[CHCl_2]^+$ and $[C_7H_7CHCl]^+$ were formed, implicating chloroform in the ionization mechanisms with toluene [31].

Besides the influence of the vaporizer temperature, other factors such as the dopant proportion, the lamp current and the solvent flow rate presented an incidence on the ionization efficiency of the analytes. For example, with the PhotoSpray, signals reached a maximum at 10% of added dopant. Regarding the lamp current, 0.8 mA was the value allowing the highest efficiency whatever the source [2;80]. Considering the solvent flow, as in the dopant-only situation, the ionization of acridine and 9-methylanthracene diminished with both sources as a function of the solvent flow rate. Ionization was higher between 50 $\mu L \cdot min^{-1}$ and 100 $\mu L \cdot min^{-1}$ [3;26]. Kauppila *et al.* showed that sensitivity loss at high solvent flow rates was predominant for charge transfer, whatever the source, but had also an deleterious effect on proton transfer [26]. This decrease in $[M+H]^+$ ions at high solvent flow rate was attributed to in-source formation of solvent-solvent or dopant-solvent clusters when using polar solvents such as methanol, acetonitrile or water (see also sections 3.1 and 3.2). Different sizes of clusters were observed, and the population of each cluster size was dependent on the temperature of the vaporizer, on the strength of the solvent-dopant bonds and especially on the solvent concentration, indicating that high solvent flow rates promoted the formation of large clusters. The loss and the gain of solvent molecules in the clusters were found to be in equilibrium. Ionization of low PA analytes by proton transfer was not efficient since large, protonated clusters were stabilized by a high solvation energy (SE). Proton transfer was therefore almost inefficient at high solvent flow rates. These large, protonated clusters can undergo two neutralization mechanisms. Moreover, the effect of the solvent flow rate on the ionization appeared to be compound dependent. For example, acridine (relatively high PA and SE) possessed a minor loss in sensitivity with an increase in the solvent flow rate compared with 9-methylanthracene (lower PA and SE) under the same conditions [31].

Furthermore, Robb and Blades showed that DA-APPI could be used for the simultaneous ionization of polar and non-polar compounds [104]. Indeed, by using toluene as a dopant and by applying a low solvent flow rate, toluene photoions remained in the source and ionized the non-polar compounds by charge transfer, while the polar molecules were preferentially ionized by the protonated solvent molecules via proton transfer. For example, by decreasing the mobile phase (water/methanol 1:1) flow rate from 200 $\mu L \cdot min^{-1}$ to 50 $\mu L \cdot min^{-1}$, ionization by charge exchange increased by a factor of ten [104].

However, the use of a dopant in APPI has also been shown to have, in some cases, a deleterious effect on the ionization performance. Hanold *et al*. observed that, in the case of the ionization of fat-soluble vitamins (A, D_2, E and K_1) with the PhotoMate source, toluene could enhance the background noise, leading to a decrease of the S/N ratio. However, both the analyte signal and the background noise were enhanced with acetone [2].

Finally, two studies compared the benefit obtained by either single dopants or mixtures of dopants. Itoh *et al.* in 2006 [60] evaluated the use of acetone, toluene, anisole and six mixtures of toluene and anisole on the ionization of PAH with an APPI-2010 source (Shimadzu) , and found that a 99.5:0.5 toluene/anisole mixture gave the best sensitivity (around 4 to 40 times greater that that found using pure toluene). Himmelsbach *et al.* also investigated the efficiency of a 1:1 toluene/acetone mixture for the ionization of various drugs [52]. This mixture did not increase signals compared to

either toluene or acetone alone. The advantage of dopant mixtures largely depends on the analytes, the dopant proportions and their natures.

4.3 Anisole

To overcome the problem of the sensitivity loss in presence of acetonitrile whatever the dopant, Kauppila *et al.* proposed anisole as an efficient dopant for charge transfer using the PhotoSpray source [24]. It was observed that anisole mostly promoted the formation of molecular ions, whereas toluene generated several protonated molecules. The analytes' ionization mechanisms mainly depended on the nature of the molecules. Indeed, carbamazepine, previously used by Robb *et al.* [1], exhibited both $[M+H]^+$ and $[2M+H]^+$ ions when infused in water/acetonitrile with toluene (in agreement with the study presented in section 4.2 [21;34]). Similar observations were made with luteolin and catechin using toluene, whereas $[M+H]^+$ and $[2M+H]^+$ ions were replaced by $[M]^{+\bullet}$ ions with anisole.

Globally, intensities of the $[M+H]^+$ ions obtained with toluene were comparable to $[M]^{+\bullet}$ intensities achieved with anisole. For compounds with low PA and IE that are still ionized with toluene [1], such as 2-naphthol, anthracene and diphenyl sulfide, anisole appeared to be more efficient and produced more intense signals (*ca.* 100 times) [24]. However, charge exchange became ineffective for compounds with higher IE values than anisole. Testosterone was for example poorly ionized due to its high IE value.

4.4 Tetrahydrofuran and hexafluorobenzene

Only two papers related to the use of other dopant molecules such as tetrahydrofuran [36] and hexafluorobenzene [35]. However, no significant performance enhancement was observed. The ionization of flavonoids with tetrahydrofuran decreased by a factor of two relative to the use of toluene [36], and hexafluorobenzene was shown to provide similar signal intensities than both toluene and anisole for the ionization of the hexamethonium bromide salt in aqueous solutions [35].

As a conclusion, toluene was found to be the most effective dopant for proton transfer while charge transfer was less favored. In the latter case, anisole was the most helpful dopant and therefore complementary to toluene. Other dopants were found to be less useful for various reasons. Indeed, acetone promoted only proton transfer, but less efficiently than toluene. Benzene presented performance similar to toluene, but due to health issues, its use is not recommended and, finally, tetrahydrofuran and hexafluorobenzene did not present interesting properties.

5. APPI in the negative mode

Only a small number of papers have investigated negative ionization in APPI [2;31;36;64;94;106], where three major mechanisms [31], namely charge exchange (CX, (15)), electron capture (EC, (16)) and proton transfer (analyte deprotonation, (17)) were evidenced :

$$M + C^- \rightarrow M^- + C \quad (15)$$

$$M + e^- \rightarrow M^- \quad (16)$$

$$M + C \rightarrow [M-H]^- + [C+H]^+ \quad (17)$$

Recently, Song *et al.* discussed another ionization mechanism, anion attachment (AA), as a potential chemical reaction for molecules not ionized by EC, CX or proton transfer. AA results in hydrogen bonding, dipole attraction and polarization [94]. With the PhotoMate, Basso *et al.* showed in 2003 that negative ionization was mainly due to the generation of slow electrons produced by the irradiated, stainless steel surface in front of the krypton lamp [106]. This mechanism was investigated one year later by Hanold *et al.* [2]. The ionization almost disappeared when the metallic surface was covered with a dielectric plate in order to avoid the production of slow electrons with an energy around 3 eV (E_{lamp} (10.6 eV) - IE_{Fe} (7.7 ev) = 2.9 eV). This was confirmed with the replacement of the dielectric surface by glass, thereby maintaining the electric fields in the source.

Negative APPI generated less background noise than the positive mode [36] and was found to ionize a wider range of molecules than other negative API sources [94]. On the other hand, Kauppila *et al.* observed the ionization of only 3 of 7 naphthalenes [31]. The addition of toluene as a dopant produced a tremendous signal increase, indicating that the ionization was initiated by thermal electrons originating from toluene ions [2;31].

Oxidation products were also observed to be in abundance, leading to the conclusion that oxygen present in the source plays an important role. As an example, oxygen (O_2), with an EA of 0.45eV, was ionized in $O_2^{\bullet-}$ by the capture of thermal electrons and subsequently ionized 1,4-naphthoquinone (EA,1.8eV) *via* various reactions due to the difference in their EA values. These oxidation products were more abundant than the expected $[M]^{\bullet-}$, particularly in presence of methanol. For 2-naphthol ionization, $[M-H]^-$ was probably formed *via* proton transfer from 2-naphthol (possessing an acidic group) to $O_2^{\bullet-}$ (which is a strong base in the gas-phase [107]). These oxidation products were not associated with the dopant proportion or the applied voltage between the source block and the curtain plate, and they obviously originated from trace oxygen in the ionization source. The latter was searched for, but neither oxygen dissolved in the solvents nor oxygen originating from the solvent molecules breaking down in the source was responsible for the formation of the oxidation products. The influence of the nebulizing gas was therefore evaluated, and the presence of a low quantity of oxygen (1 ppm) could induce oxidation [31;64]. Kauppila *et al.* evaluated its influence by the use of high purity nitrogen (99.999%) or by purified air as nebulizing gases with the PhotoSpray. It was found that, with high quality nitrogen, $[M]^{\bullet-}$ and $[M-H+O]^-$ ions were in a relative abundance of

100% and 81%, respectively, whereas with air, $[M]^{\bullet-}$ decreased to 48%, and $[M-H+O]^-$ increased to 100%.

The addition of an acid (acetic) or a base (ammonium hydroxide) to the mobile phase hampered the formation of both 1,4-naphthoquinone and 2-naphthol $[M]^{\bullet-}$ ions; furthermore, it decreased the abundance of the oxidation products, which was probably due to the consumption of thermal electrons by H^+ to form H_2 and/or by NH_4^+ to form NH_3 and H_2 [31;36;94]. Nevertheless, Rauha *et al.* observed higher signal intensities with ammonium hydroxide, in the analysis of glycosides (flavonoids) [36]. The addition of a salt (ammonium acetate) inhibited the formation of $[M]^{\bullet-}$ without influencing the oxidation products [31;36]. For compounds with gas-phase acidity higher than $O_2^{\bullet-}$ and lower than toluene, Song *et al.* showed that the presence of di-*tert*-butyl peroxide improved analyte deprotonation *via* the formation of a basic anion generated from toluene, $C_6H_5CH_2^-$ [94]. Due to the capture of thermal electrons, ionization was suppressed for naphthalenes with low, gas-phase acidity by halogenated solvents (chloroform and TFA). For naphthalenes with high gas-phase acidity, $[M-H]^-$ ions were observed [64].

6. APPI vs ESI, APCI and dual sources

APPI appears to be a good alternative to ESI and APCI for the coupling of low flow rate separation techniques (< 50 $\mu L \cdot min^{-1}$, see section 06.3) such as CE and capillary LC, due to the lower concentration of the solvent vapor in the ion source. Furthermore, APPI is generally considered to be a compatible ionization technique for apolar compounds. In fact, APPI was found by various authors to be more complementary to ESI than APCI, due to its ability to ionize molecules with lower polarity than can be ionized in APCI.

While several authors have directly compared the performance of the various available API sources on a variety of analytical compounds, others have tried to implement a dual source strategy. The latter principle was first reported in 1996 [108] with the electron ionization / chemical ionization (EI/CI) for GC-MS applications. Using a dual-source may expand the range of ionizable molecules during a single chromatographic run. The dual mode generally gave lower sensitivities and better repeatability than running each ionization mode separately [3].

6.1 APPI compared to ESI

APPI and ESI are relatively orthogonal ionization sources with regards to the range of tested compounds [3]. Therefore, depending on the analytes and the separation conditions, APPI and ESI can either be complementary or one can be more appropriate than the other. For example, APPI was more efficient for olive oil identification in ionizing mono- and diacylglycerols while ESI was better for triacylglycerols [93]. For the analysis of unconjugated hormones in river water, APPI showed a better

sensitivity than ESI, but the opposite situation was encountered for conjugated hormones [86]. In numerous cases, APPI was more sensitive than ESI, as in the analysis of aflatoxin M1 in milk [109], trifluorobenzoic acid [2], lipids (with cleaner MS spectra) [110], amininitropyrene and dinitropyrene [74] and estrone (steroid) [3]. In this case, the ionization intensity was more than one order of magnitude higher in APPI. Ionization performance was also compared *via* an analysis of 201 drugs with a wide range of chemistry, and APPI ionized 98% of the compounds of the set whereas ESI only produced ions in 91% of the cases [81]. APPI also provided a wider, linear dynamic range (3-4 orders of magnitude versus 2-3 for ESI) [68]. Finally, ESI was more sensitive for the analysis of cyclosporin A in rat plasma [73], anabolic steroids (even using a buffered mobile phase) [42], and flavonoids [36], and it probably remains the most adaptable source for the ionization of charged compounds, such as apomorphine, dobutamine, and entacapone phase II thermolabile metabolites in biological samples. Indeed, ESI allowed for the detection of 22 molecules, whereas APPI only helped to detect 14, which was due to the lower probe temperatures applied in ESI [66].

6.2 ESI/APPI dual mode

Progesterone and testosterone were used to evaluate the ionization efficiency of the ESI/APPI dual source [3]. In a single ESI, only low intensity signals of multiple ions were observed, which disappeared in the dual mode, while abundant protonated molecules were formed in both the single and the dual-mode APPI. The ESI/APPI combination was shown to be very efficient in the simultaneous analysis of testosterone and horse myoglobin. ESI was more effective in protein (myoglobin) ionization, whereas APPI was found to be particularly adapted for testosterone. Similar results were described in the analysis of melittin and 2,7-dibromo-9,9-bis(2-ethylhexyl)fluorine [3]. Therefore, the dual ESI/APPI interface would be very useful, for example, in clinical diagnostics for the concomitant determination of pharmaceutical molecules with various polarity and protein and drug/macromolecule interactions. This concept was recently explored by Short and Syage [111], who have published a study using an electrospray photo-ionization (ESPI) source for the simultaneous analysis of low polarity (pharmaceutical, hormone or sterol) and polar compounds (cyclodextrin). The ESPI source provided several advantages over the separate use of both modes, including better performance at low flow-rates. Data acquisition was performed by alternating the ESI and APPI modes. In comparison to electrospray chemical ionization (ESCI), ESPI possessed better S/N ratios for all of the studied complexes of cyclodextrin (naproxen, flurbiprofen, nimesulide, progesterone and cholesterol).

6.3 APPI compared to APCI

One important difference between APPI and APCI concerns their ionization efficiency *versus* the mobile phase flow rate. APPI worked properly below 50 $\mu L \cdot min^{-1}$, while better results are obtained

with APCI for larger values (> 1 mL·min^{-1}, for mechanistic considerations, see sections 2 and 4). Despite APPI is commonly considered as a mass flow sensitive detector, it behaves like a concentration flow sensitive detector at flow rates below 50 µL·min^{-1}. The lower sensitivity of APPI at high flow rates could not be compensated by an increase of either the dopant flow or the discharge lamp current [80].

For values comprised between 50 µL·min^{-1} and 1 mL·min^{-1}, the APPI signal was found to be directly proportional with the flow rate. The APPI ionization was, therefore, believed to be a first-order process in the analyte mass (a mass-dependent process) and a zero-order process in the solvent mass, suggesting that the methanol solvent was not involved in the photoionization process. On the other hand, APCI was dependent on both the solvent and analyte density since a CI process depends on the presence of solvent for charge transfer, thus explaining the loss in sensitivity at low solvent flow rates [2]. For high PA compounds, however, Kauppila *et al.* did not observe any significant decrease in the ion production with both APPI sources (Sciex prototype or Agilent/Syagen PhotoMate) [26], indicating that proton transfer is less dependent on the solvent flow rate compared to charge transfer.

Generally, APPI ionized more compounds (98%) than APCI (91%) among the set of 201 molecules previously cited [81]. In the positive mode, the dynamic range measured for reserpine with the PhotoMate source was about 1 pg to over 100 ng, comparable to APCI [2]. Signal stability was excellent, with an RSD less than 3% for 600 injections over a 15h time span. With toluene as a dopant and methanol as a chromatographic solvent, the $[M+H]^+$ ions of carbamazepine and acridine were found to be about 8 times higher in APPI than in APCI, whereas no molecular ions were observed in APCI. With toluene and acetonitrile, APPI was only slightly better than APCI for charge transfer but was significantly better for proton transfer [1]. Estrone ionization was also about 5 times higher in APPI than in APCI, and naphthalene photoionization was less sensitive to the solvent conditions than was APCI [3]. Besides, Cai *et al.* observed in normal-phase LC that APPI provided better repeatability and higher signal to noise ratios than APCI, which led to better sensitivities depending on the analyte (a factor 3 to 530). As expected, APPI was found to be particularly suitable when using a flammable solvent such as hexane and offered a decisive advantage compared to APCI with regards to potential explosions. Besides, APCI generates a deposit of carbon on the corona discharge needle and on the cone face, which can lead to instabilities and a decrease in signal over time [56].

6.4 APCI/APPI dual mode

Syage *et al.* demonstrated that the dual mode can induce signals with intensities different from the sum of both single modes [3]. This suggested that either positive or negative interferences can occur when running both modes simultaneously. For example, the $[M]^{\bullet+}$ naphthalene ions were produced in a single APPI, whereas they disappeared when APCI was simultaneously operated. The mechanism was not elucidated but certainly involved a large number of free electrons generated by

the corona discharge needle, thereby neutralizing the positive charges. For injections of hydrocortisone between 1 pg and 1 ng, the three modes (APCI, APPI and APCI/APPI) possessed a similar, linear domain. The hydrocortisone signal was found to be slightly higher with APPI than with APCI, whereas its ionization in the dual-mode produced more signal than the sum of independent analyses.

7. Matrix effects

Due to the principle of APPI, photoionization should not be subject to interfering mechanisms in the analytes' ionization [2]. However, ion suppression could occur through proton transfer from the ionized analyte to other molecules present in the source (such as the solvent, matrix and endogenous compounds). Solvents with low IE values, such as DMSO, could transfer a charge from the ionized analyte to the solvent molecule. Solvents with high PA values could operate the same way by proton transfer through two possible mechanisms :

$$[M+H]^+ + S \rightarrow M + [S+H]^+ \quad (18)$$

$$[M]^{\bullet+} + S \rightarrow [M-H]^{\bullet} + [S+H]^+ \quad (19)$$

In 2004, Hanold *et al.* compared the effect of endogenous compounds from rat plasma on the ionization of fluphenazine with ESI, APCI and APPI. ESI possessed more significant ionization suppression in comparison to both APCI and APPI [2].

Another study compared ESI, APCI and APPI ionization sources with regards to matrix effects arising from endogenous compounds in human plasma by using a dedicated set-up inspired from Bonfiglio *et al.* [112]. The PhotoMate source was observed to be the least sensitive to matrix effects on the analysis of basic analytes as model compounds. APPI provided ion enhancement, indicating a possible dopant effect of the endogenous compounds [57]. The authors concluded that the choice of ionization source should be made by evaluating the best compromise between the achievable sensitivity and any encountered matrix effects.

8. CE-APPI/MS

The parameters influencing CE-APPI-MS were investigated by Schappler *et al.* with an experimental design methodology using a sheath-liquid interface [113]. It was observed that with sheath-liquid flow, the drying gas flow and the vaporizer temperature were the most important parameters for the detection of amphetamines. Other experimental parameters, such as the nebulizer pressure, capillary voltage and drying gas temperature, were not significant. When a dopant is

necessary, it can be added to the sheath-liquid [43;45]. By adjusting the position of the nebulizer, a 10-fold increase in the signal intensity could be obtained [114]. As already mentioned in section 06.1, the use of APPI in miniaturized techniques such as CE-MS is particularly interesting due its compatibility with low flow rates and commonly used background electrolytes (BGE). Non-volatile BGE did not induce any signal alterations in APPI, thereby resulting in a wider choice of CE buffers when transferring analytical conditions from CE-UV to CE-MS [43;45;54;114;115]. For the analysis of labetolol, salbutamol and terbutaline, both borate and phosphate BGE provided better signal/noise (S/N) ratios in APPI than in ESI, as demonstrated by Hanold *et al*. [2]. Higher abundances and an important reduction of the background noise were also observed, particularly with the phosphate buffer, while the formation of borate clusters contributed to higher noise. The ammonium buffer was shown by Nilsson *et al.* to have a deleterious effect on the toluene DA-APPI of naphthalenes, due to the high PA of ammonium ions [114].

Sodium dodecyl sulfate (SDS) did not lead to signal alteration in APPI [43;45], which is particularly important for coupling microemulsion electrokinetic chromatography (MEEKC) and micellar electrokinetic chromatography (MEKC) with APPI-MS. The latter allowed the simultaneous separation and detection of charged/polar and neutral/apolar compounds in a single run. This approach could be very useful in sample profiling, where unknown analytes with various physico-chemical properties are analyzed [45]. In the case of the analysis of drugs as standards, Himmelsbach *et al.* presented in 2007 the on-line coupling of MEEKC-APPI-MS for the determination of nine drugs in water [116]. The limits of detection (LOD) ranged between 0.5-5.0 $\mu g \cdot mL^{-1}$. The same group also presented a MEEKC-APPI-MS/MS method for the analysis of eight moderately polar drugs in water [52]. The LOD comprised between 3-41 $\mu g \cdot mL^{-1}$ in a single MS and between 0.6-6.0 $\mu g \cdot mL^{-1}$ in MS^2 were achieved. Moreover, other MEEKC formulations were tested (oil-phase buffer systems and other surfactants) without any interferences on the MS detection. On the other hand, Schappler *et al.* found that the compatibility of SDS with APPI depended on the analytes and the selected polarity. Indeed, MEEKC-APPI-MS used in the negative mode for the analysis of various diuretics showed a 15 to 20-fold loss in sensitivity compared to CZE-ESI-MS, which can be mainly attributed to the presence of $[SDS\text{-}Na]^-$ adduct [117].

An interesting feature was observed with the PhotoMate source by Hommerson *et al.* [115]. Non-polar compounds with low PA were preferentially ionized by APPI or DA-APPI, whereas quaternary ammonium compounds were found to generate higher sensitivities when the lamp was switched off [115]. This phenomenon was attributed to an ESI-like liquid phase ionization, and it existed only for very specific conditions, such as when the capillary voltage was set to a low value (600V).The same authors compared ESI and APPI for the analysis of basic amines, quaternary amines and steroids. A moderate ion suppression in the ESI with phosphate buffers was found, while no signal alteration in the APPI was observed. Nevertheless, sensitivities achieved in ESI-MS for basic amines were better than in APPI-MS by a factor of 2 to 5. Steroids presented similar signal intensities with both sources.

9. Applications

Articles appearing after the publications of the previous reviews [22;23] have been considered, covering the time period from January 2005 to April 2008. Among the studies published in this period, several classes of analytes emerged: drugs [44;51-53;61;73;78;86;98;116;118-120] (Table 3), lipids [46;47;87;91;110;121;122] (Table 4), natural compounds [48;49;82;85;93;95;109;123-125] (Table 5), pesticides [83;101] (Table 6), synthetic organics [58;92;97;100;103;126-131] (Table 7) and petroleum derivatives [96;132;133] (Table 8).

9.1 Drugs

The majority of APPI applications were dedicated to the determination of drugs in biofluids, such as serum [78;119] and plasma [53;61;73;98;120]. Serum was investigated by Lembcke *et al*. [78], who proposed an LC-APPI-MS/MS method for the analysis of phytosterols in 15 min instead of in GC-MS, which required about 3h. The LOD ranged between 0.25-0.68 $\mu g \cdot L^{-1}$. Another LC-APPI-MS/MS application was proposed by Martens-Lobenhoffer *et al.* [119] for the stereoselective quantification of omeprazol and its two major metabolites (5-hydroxyomeprazole and omeprazole sulfone) in serum. The analytical range was between 5-750 $ng \cdot mL^{-1}$ for both omeprazole and omeprazole sulfone and was between 2.5-375.0 $ng \cdot mL^{-1}$ for the 5-hydroxy- metabolite.

Regarding the analysis of drugs in plasma, Wang *et al.* compared five reversed-phase and normal-phase LC-MS methods using ESI, APCI and APPI sources for the analysis of cyclosporine A in rat plasma [73]. Normal-phase LC-APPI-MS was very efficient in terms of ionization since solvents used in the mobile phase (such as isooctane and ethanol) promoted the analytes' ionization. Hsieh *et al.* determined cladribine and clofarabine in plasma (mouse) [53]. Different fast separation techniques, including UHPLC, coupled to various MS systems (linear ion trap and triple quadrupole MS) equipped with APPI or APCI were evaluated. No significant matrix effects or ionization differences were observed. APPI was used by Theron *et al.* to overcome large matrix effects in ESI for the analysis of venlafaxin and its major metabolite (*O*-desmethylvenlafaxine) in human plasma [98]. Indeed, the use of APPI resulted in negligible ion suppression. Validation of the method was performed in the 2.4 - 605.0 $ng \cdot mL^{-1}$ range. Silva *et al.* presented a LC-APPI-MS/MS method for the analysis of isosorbide-5-mononitrate in human plasma for a bioequivalence study of tablet formulations [61]. The calibration curve was linear in the 20-2000 $ng \cdot mL^{-1}$ domain. Repeatability was 7.9% at 60 $ng \cdot mL^{-1}$, 5.2% at 300 $ng \cdot mL^{-1}$ and 7.0% at 1800 $ng \cdot mL^{-1}$, with a trueness of 94.6%, 94.1% and 88.8%, respectively. Dos Santos Pereira *et al.* proposed a LC-APPI-MS/MS method for the quantification of betamethasone in human plasma [120]. The calibration curve was linear in the 0.05-50 $ng \cdot mL^{-1}$ domain, and the repeatability was lower than 12% for all tested concentration levels with a relative bias lower than 10 %. Drugs have also been analyzed in food. Mohamed *et al.* validated a LC-APPI-MS/MS method for the analysis of 16 antibiotics in honey, with a LOD in the 0.4-4.5 $\mu g \cdot Kg^{-1}$ range for a targeted concentration of 50 $\mu g \cdot Kg^{-1}$ [51].

Turnipseed *et al.* compared ESI, APCI, APPI and APCI/APPI ionization methods for the analysis of anthelmintics in cow milk [44]. As expected, the MS responses varied as a function of the source and of the analytes. The best results were found when running the dual APCI/APPI source in the APPI-only configuration.

Only two applications of APPI in the field of compounds naturally present in the human body, such as hormones or neurotransmitters, have been published during the 2005-2008 period (see Table 4). The first application was dedicated to human hormones present in water. Yamamoto *et al.* compared ESI and APPI for the analysis of steroids in Osaka City rivers and estuaries [86]. ESI was found to provide better sensitivities for conjugated steroids, whereas APPI was better in ionizing unconjugated steroids. Estrone, estrone 3-sulfate and 4-androstene-3,17-dione were observed at maximal concentrations of 51 $ng \cdot L^{-1}$, 5.1 $ng \cdot L^{-1}$ and 6.4 $ng \cdot L^{-1}$, respectively. The LODs were in the 0.06-7.0 $ng \cdot L^{-1}$ range, depending on the analyte. The second paper presented a LC-APPI-MS method for the quantitation of salsolinol and related catecholamines in rat brain tissue [118]. The calibration curve presented an R^2 value above 0.96 for over two orders of magnitude.

Table 3 : Summary of APPI applications for the analysis of drugs.

analyte	matrix	source	dual source	dopant	proportion	polarity	ref
stigmasterol β-sitosterol campesterol brassicasterol cholesterol cholesteryl stearate	human serum	PS	-	toluene	15%	P	78
omeprazole 5-hydroxyomeprazole omeprazole sulfone	human serum	PM	-	none	-	P	119
isosorbide-5-mononitrate	human plasma	PS	-	acetone	5%	N	61
betamethasone	human plasma	PS	-	toluene	3%	N	120
venlafaxine O-desmethylvenlafaxine	human plasma	PS	-	toluene	36%	P	98
cladribine clofarabine ketoconazole	mouse plasma	PS	-	none acetone toluene THF	- 17% 17% 17%	P	53
cyclosporin A	rat plasma	PS	-	toluene	2%	P	73
ivermectin doramectin eprinomectin milbemycin moxidectin	cow milk	PM	APCI/APPI	acetone	5%	P/N	44
sulfanilamide dapson sulfapyridine sulfadiazine sulfamethoxazole sulfathiazole sulfamerazine sulfamoxole	honey	PS	-	toluene	10%	P	51

sulfabenzamide sulfisomidine slfamethiazine sulfamethoxypyridiazine sulfameter sulfachlorpyridiazine sulfaquinoxaline sulfadoxine sulfadimethoxine sulfasalazine							
pentoxifylline trimethoprim phenacetin metoprolol carbazepine trazodone diltiazem clomipramine	standard	PM	-	toluene	n.a.	P	52
pentoxifylline phenacetin 4-dimethylaminobenzaldehyde quinoline isoquinoline metoprolol carbamazepine 3-methylquinoline 6-methylquinoline	standard	PM	-	acetone	5%	P	116
dopamine salsolinol 3,4-dihydroxybenzylamine norsalsolinol tetrahydropapaveroline norepinephrine epinephrine	rat brain tissue	PM	-	acetone	7.5%	P	118
testosterone 4-androstene-3,17-dione androsterone epiandrosterone dehydroepiandrosterone (DHEA) 5α-dihydrotestosterone estrone 17β-estradiol estriol dehydroepiandrosterone-3-sulfate estrone-3-sulfate 17β-estradiol 3-sulfate estriol 3-sulfate estrone-3-β-D-glucuronide 17β-estradiol 3-β-D-glucuronide estriol 3-β-D-glucuronide estrogen ethynylestradiol	environmental water	PS	-	toluene	25%	P/N	86

P : positive polarity
N : negative polarity

P/N : positive and negative polarities
PS : PhotoSpray source
PM : PhotoMate source

9.2 Lipids

This class of compounds was of particular interest for APPI due to their low polarity. Cai *et al*. compared ESI, APCI and APPI for the normal-phase LC-MS analysis of free fatty acids in fish oil and their esters, monoglyceride, diglyceride and triglyceridel, which are usually analysed by reversed-phase chromatography [110]. APPI provided the lowest LOD and the highest S/N ratios. It was 2-4 times more sensitive than APCI or ESI. APPI's linear dynamic range was found to be 4-5 orders of magnitude with an upper limit around 1250-2500 ng. This was confirmed in another study concerning the analysis of fatty acids and acylglycol lipids [121]. APPI provided very clean MS spectra, which were stable and repeatable. It also appeared that non-aqueous mobile phases provided better sensitivities and peak shapes than aqueous mobile phases, which required higher vaporizer temperatures for the analyte desolvation. Low vaporizer temperatures were found to significantly reduce the analyte's thermal decomposition, and the signals were, therefore, improved. The cone voltage was determined as a key parameter determining the sensitivity of this method. The on-column LOD was estimated to 94 pg for an EPA methyl ester, 90 pg for monoarachidin and diarachidin and 24 pg for trielaidin. Roy *et al*. proposed a porous, graphitic carbon LC-APPI-MS/MS method for the analysis of glycosphingolipids, thereby providing structural information on the fatty acid and sphingoid base [47]. This set-up allowed for the efficient discrimination of isobaric compounds. In the paper of Delobel *et al*., a LC-APPI-MS method was proposed for the analysis of globotriaosylceramides in human urine, which are connected with Fabry disease [46]. Collision-induced dissociation (CID) in the positive mode provided very useful information for the determination of alkyl chains structures. Another concern about the use of APPI for the analysis of lipids was proposed by Müller *et al*. [87]. They suggested a (Ag+)-HPLC-APPI-MS/MS method for the analysis of conjugated linoleic acid's elongation and β-oxidation products usually operated in GC-MS. The use of APPI provided a 40-fold better sensitivity than APCI and allowed for the determination of the double bond positions, the configurations and the chains lengths of the conjugated linoleic acids. Finally, a LC-APPI-MS/MS method was proposed by Muñoz-Garcia *et al*. for the identification of sphingolipids mixtures in the stratum corneum of house sparrows [91;122]. Seven families of cerebrosides (97 compounds) and four families of ceramides (79 compounds) were brought out, for which fatty acids carbon chains typically contained 40 carbons, longer than those generally reported for mammalian stratum corneum.

Table 4 : Summary of APPI applications for the analysis of lipids.

analyte	matrix	source	dopant	proportion	polarity	ref
eicosapentaenoic acid methyl ester monoarachidin diarachidin docosahexaenoic acid trielaidin	standard	PM	none	-	P/N	121
glucosylceramide galactosylceramide lactosylceramide globoside globopentaosylceramide globotriaosylceramide	standard	PS	acetone	25%	P/N	47
eicosapentaenoic acid methyl ester monoarachidin diarachidin trielaidin	fish oil	PM	none	-	P/N	110
globotriaosylceramides	urine	PS	acetone none	12.5 & 25% -	P/N	46
conjugated linoleic acids	human coronary artery smooth muscle cells	PS	toluene	5%	P	87
cholesterol free fatty acids ceramides cerebrosides	house sparrows stratum corneum	PS	toluene	1%	P/N	91
water sphingolipids	house sparrows stratum corneum	PS	toluene	1%	P/N	122

P : positive polarity
N : negative polarity
P/N : positive and negative polarities
PS : PhotoSpray source
PM : PhotoMate source

9.3 Natural compounds

The use of APPI was also investigated for the analysis of natural, low polarity compounds. For example, Gómez-Ariza *et al.* have published two studies on APPI performance, one for an olive oil analysis and one for the classification of unknown wine samples [85]. In the case of olive oil authentification, the authors first compared ESI and APPI for LC-MS analysis [93]. Both sources were found to be complementary to one another, as APPI was more sensitive for the monoacyl- and diacylglycerols and ESI for triacylglycerols. Flow injection analysis-APPI-MS, using a QqTOF apparatus, was used in a complementary fingerprinting study. This method was able to discriminate olive, extra-virgin olive, olive-pomace, hazelnut, sunflower, corn and several mixed oils in about one minute per sample [82]. The same apparatus was also used for wine classification according to anthocyanins profiles [85]. Performance of ESI and APPI sources was also compared by Cavaliere *et al.* for the LC-MS/MS analysis of aflotoxins M1 and B1 in cow milk [109] and by Pardo *et al.* for the LC-MS/MS analysis of domoic acid in shellfish [124], who included evaluation of APCI and APCI/APPI sources. Both groups selected ESI either due to its lower LOD [124] or to its higher robustness [109]. Ergosterol quantitation was operated by Varga *et al.* with an LC-APPI-MS method to evaluate the fungal biomass in wheat [49]. The calibration curve was linear between 0.05-50 ng·µL^{-}

[1] with a repeatability value between 2.1% and 5.2%. Concerning the elucidation of the straw lignin molecular structure, Banoub *et al.* found the presence of 57 specific oligomers with an LC-APPI-QqTOFMS apparatus [95]. The latter was demonstrated to be a powerful analytical tool for the ionization of small and large lignin oligomers. The paper of Cao *et al.* compared ESI, APCI and APPI sources for the rapid LC-MS analysis of melatonin, serotonin and auxin in plants [123]. The performance was comparable, and a LOD was found to be 0.2 $ng \cdot mL^{-1}$ for melatonin, 5 $ng \cdot mL^{-1}$ for serotonin and 0.7 $ng \cdot mL^{-1}$ for auxin. The linear dynamic ranges were 0.02 $ng \cdot mL^{-1}$ - 0.1 $mg \cdot mL^{-1}$, 5 $ng \cdot mL^{-1}$ - 0.1 $mg \cdot mL^{-1}$ and 0.7 $ng \cdot mL^{-1}$ - 0.1 $mg \cdot mL^{-1}$, respectively. Tonidandel *et al.* classified various samples of amber with the help of laser desorption ionization (LDI), APCI and APPI-ionization techniques with multivariate data analysis [125]. APPI was the most suitable ionization mode for the analysis of amber, as fossilization produced highly stable, insoluble polymers. Finally, Jean-Denis *et al.* proposed a rapid screening of stilbenes in grapevine leaves infected by *P. viticola* by LC-APPI-MS^n, which required only 1-2 mg of sample [48].

Table 5 : Summary of APPI applications for the analysis of natural compounds.

analyte	matrix	source	dual source	dopant	proportion	polarity	ref
anthocyanins	red wine	PS	-	toluene	12.50%	P	85
various	olive oil	PS	-	toluene	10%	P	93
various	olive oil	PS	-	toluene	10%	P	82
aflatoxin M1 aflatoxin B1	cow milk	PS	-	none	-	P	109
ergosterol	wheat	PM	-	toluene	1%	P	49
wheat straw lignin polymer	wheat	PS	-	toluene	n.a.	P/N	95
trans-resveratrol trans-ε-viniferin α-viniferin trans-δ-viniferin trans-pterostilbene	grapevine leaves	PM	-	acetone	n.a.	N	48
serotonin melatonin indole-3-acetic acid	higher plants	PM	-	none	-	P	123
domoic acid	shellfish	PM	APCI/APPI	none	-	P	124
amber	amber						125

P : positive polarity
N : negative polarity
P/N : positive and negative polarities
PS : PhotoSpray source
PM : PhotoMate source

9.4 Pesticides

Within the selected time period, only two papers reported analyses of pesticides using APPI, and both were applied to surface and ground water determinations of DDT [101] and metolachlor [83]. For the former, a linear dynamic range of the method was found between 20-1000 $ng \cdot mL^{-1}$ with a LOD of 7 $ng \cdot mL^{-1}$, higher than the LOD achieved by conventional GC-MS. For metolachlor diastereoisomers and s-metolachlor analysis, Kabler and Chen proposed an enantioselective LC-APPI-MS/MS method with a LOD of 0.10 ppb, with a repeatability lower than 20% [83].

Table 6 : Summary of APPI applications for the analysis of pesticides.

analyte	matrix	source	dopant	proportion	polarity	ref
diastereoisomers of metolachlor	environmental water	PS	toluene	1%	P	83
p,p'-dichlorodiphenyltrichloroethane (DDT)	environmental water	PS	toluene	5%	P/N	101

P : positive polarity
P/N : positive and negative polarities
PS : PhotoSpray source

9.5 Synthetic organics

Among the papers published for the analysis of organic compounds, four concerned the determination of flame retardants [126;129-131]. In case of brominated substances, Riu *et al.* proposed an LC-APPI-MS^n as an alternative to the generally used GC-EI/MS method [126]. Br_2 loss was the major ionization process for polybrominated flame retardants, and the ionization polarity modes were found complementary with regards to the degree and position of bromination. The positive mode favored the loss of Br_2 for the ortho-subsituted compounds and $COBr^{\bullet}$ for the non ortho-subsituted compounds, while the loss of both Br_2 and HBr was observed in the negative mode. The second study presented the determination of decabromodiphenyl ether in rat tissue [131], which allowed the identification of a very toxic derivative, namely, hydroxylated octa-decabromodiphenyl ether, in liver. The third paper was dedicated to the analysis of tetrabromobisphenol A and polybromodiphenylethers [130]. The former was well ionized in the negative mode without dopant, while the positive mode was preferentially used for the mono- to penta-polybromodiphenylethers. For higher bromo-substitutions, the negative mode provided $[M-Br+O]^-$ ions, required toluene as dopant, and was finally selected. To finish, Davis *et al.* proposed the identification of α-, β- and γ-hexabromocyclododecane metabolites in both wastewater sludge and freshwater [129]. Hexabromocyclododecane was found to be sequentially debrominated with the loss of 2 bromines at each step, giving rise to tetrabromocyclododecane, dibromocyclododecane and cyclododecatriene.

Concerning the analysis of PAH, Zheng *et al.* proposed a CEC-APPI-MS method for the analysis of the 12 monomethylated benzo[α]pyrene isomers [127]. APPI, which was compared to ESI

and APCI, provided the highest signals. The method showed a linear domain of 2.5-50 $\mu g \cdot mL^{-1}$, and an LOD of 400 $ng \cdot mL^{-1}$ for the 1-, 3-, and 11-methylbenzo[α]pyrene, which was 100 times lower than the CEC-UV results. Ten carcinogenic PAH were analyzed in mainstream cigarette smoke by Ding *et al.* who developed and validated the LC-APPI-MS/MS method with an LOD in the 11-166 pg range [97]. The last investigation of PAH was run by Grosse *et al.* who proposed a µ-LC-MS method for the determination of partially oxidized PAH [58]. ESI, APPI and/or APCI ionization techniques were tested. No ionization for any compound was achieved with ESI. APPI produced mainly negative ions, whereas APCI was found to be best adapted for the ionization of partially oxidized PAH since it produced both positive and negative ions. The dual mode was found to be the best regarding cost, time, the amount of sample required and structural information.

Other studies were dedicated to various compounds, such as alkanox P24 (a commercial phosphite antioxidant), nitroaromatics, perfluorinated compounds, pentafluorobenzyl derivatives and benzidine and related compounds. Alkanox P24 was analyzed by LC by Papanastasiou *et al.* to assess its hydrolysis in water [92]. APPI and APCI were compared, and APPI was selected for further investigations since it showed a lower LOD. In the positive mode, the $[M+H]^+$ ions were produced, and in the negative mode, the $[M-H]^-$ ions were predominant, and no protonated molecules were detected. Alkanox P24 was exclusively hydrolyzed by scission of both $P\text{-}O_{phenol}$ bonds, thereby yielding 2,4-di-*tert*-butyl phenol. Crescenzi *et al.* also compared APCI and APPI for on-line-SPE-LC-MS methods dedicated to the determination of nitroaromatics and related compounds at trace levels in water [128]. APCI gave better ionization for the cyclic nitramines and nitrate esters. Song *et al.* proposed a LC-APPI-MS method for the analysis of fullerenes, perfluorinated compounds and pentafluorobenzyl derivatives, where an electron capture mechanism provided very good sensitivities in the negative mode [100]. In the flow injection analysis, the LOD was 0.15 pg for fullerene and 1.5 pg for perfluoromethylcyclohexane. Finally, Bacaloni *et al.* proposed a normal-phase LC-APPI-MS/MS method for the determination of benzidine, 3,3'-dichlorobenzidine, mono-, di-, and tri-chloroanilines in water. LOQs were comprised in the 7-112 $ng \cdot L^{-1}$ range [103].

Table 7 : Summary of APPI applications for the analysis of synthetic organics.

analyte	matrix	source	dual source	dopant	proportion	polarity	ref
2,4-dibromodiphenylether 4,4'-dibromodiphenylether 2,2',4-tribromodiphenylether 2,2',4,4'-tetrabromodiphenylether 2,2',6,6'-tetrabromodiphenylether 3,3',4,4'-tetrabromodiphenylether 2,2',3,4,4'-pentabromodiphenylether 2,2',4,4',5-pentabromodiphenylether 2,3',4,4',6-pentabromodiphenylether 3,3',4,4',5-pentabromodiphenylether 2,2',3,4,4',5'-hexabromodiphenylether 2,2',4,4',6,6'-hexabromodiphenylether 2,2',3,4,4',5,6'-heptabromodiphenylether 2,2',3,4,4',5',6-	standard	PM	-	toluene	1%	P/N	126

heptabromodiphenylether 2,2',3',4,4',6,6'-heptabromodiphenylether 2,3,3',4,4',5,6-heptabromodiphenylether 2,2'3,3',4,4',5,5',6,6'-decabromodiphenylether							
1-methylbenzo[α]pyrene 2-methylbenzo[α]pyrene 3-methylbenzo[α]pyrene 4-methylbenzo[α]pyrene 5methylbenzo[α]pyrene 6-methylbenzo[α]pyrene 7-methylbenzo[α]pyrene 8-methylbenzo[α]pyrene 9-methylbenzo[α]pyrene 10-methylbenzo[α]pyrene 11-methylbenzo[α]pyrene 12-methylbenzo[α]pyrene	standard	PM	-	none	-	P	127
bis(2, 4-di-tert-butyl)pentaerythritol diphosphite (Alkanox P24)	standard	PS	-	toluene	10%	P/N	92
fullerene perfluoropentane perfluoromethylcyclohexane perfluoroadamantane hexafluorobenzene octafluorotoluene octafluoronaphthalene perfluorotributylamine pentafluorobenzyl	standard	PS	-	toluene	400%	N	100
tetrabromobisphenol A octabromodiphenylether decabromodiphenylether monobromodiphenylether	standard	PM	-	toluene	2.5%	P/N	130
1-naphthoic acid 2-naphthoic acid coumarine 4-oxa-benzo[def]chrysene-5-one 1-naphthol 2-naphthol 2-hydroxyphenanthrene 3-hydroxyphenanthrene 4-hydroxyphenanthrene 9-hydroxyphenanthrene 1-hydroxypyrene 1,5-dihydroxynaphthalene 2,7-dihydroxynaphthalene cis-4,5-dihydro-dihydroxy-benzo[a]pyrene tertralone fluoren-9-one benzanthrone 1,4-naphthoquinone 9,10-phenanthrenequinone 1,4-anthraquinone 9,10-anthraquinone 1,4-chrysenequinone benzo[a]pyrene-3,6-dione benzo[a]pyrene-4,5-dione benzo[a]pyrene-7,10-dione 5-hydronaphthoquinone 2-carboxy-9,10-anthraquinone 1-hydroxy-9,10-anthraquinone 2-hydroxy-9,10-anthraquinone	standard	PM	-	acetone	10%	P/N	58

1,8-dihydroxy-9,10-anthraquinone							
benz[a]anthracene 5-methylchrysene benzo[j]fluoranthene benzo[b]fluoranthene benzo[k]fluoranthene benzo[a]pyrene indeno[1,2,3-cd]pyrene dibenz[a,h]anthracene dibenzo[a,e]pyrene dibenzo[a,i]pyrene	cigarette smoke	PS	-	toluene	60% & 75%	P	97
hexabromocyclodecane	environmental water	PM	-	none	-	N	129
1,3,5-trinitrobenzene tetryl-2,4,6,N-tetranitrotoluene 2,4,6-trinitrotoluene 1,2-dinitrobenzene 1,3-dinitrobenzene 1,4-dinitrobenzene 2-amino-4,6-dinitrotoluene 4-amino-2,6-dinitrotoluene 2,6-diamino-4-nitrotoluene 2,3-dinitrotoluene 2,4-dinitrotoluene 2,5-dinitrotoluene 2,6-dinitrotoluene 3,5-dinitrotoluene 1,3,5-trinitro-1,3,5-triazacyclohexane 1,3,5,7-tetranitro-1,3,5,7-tetrazacyclooctane pentaerythritol tetranitrate 1,3-bis-nitrooxy-2,2-bis-nitrooxymethyl-propane	environmental water	PM	-	none	-	P/N	128
benzidine 3,3'-dichlorobenzidine 2-chloroaniline 3-chloroaniline 4-chloroaniline 2,3-dichloroaniline 2,4-dichloroaniline 2,5-dichloroaniline 2,6-dichloroaniline 3,4-dichloroaniline 3,5-dichloroaniline 2,3,4-trichloroaniline 2,4,5-trichloroaniline 2,4,6-trichloroaniline	environmental water	PS	-	toluene	15%	P/N	103
Decabromodiphenyl ether	rat tissues	Riu	APCI/APPI	toluene	1%	N	131

P : positive polarity
N : negative polarity
P/N : positive and negative polarities
PS : PhotoSpray source
PM : PhotoMate source

9.6 Petroleum derivatives

The analyses of petroleum derivatives were published by Purcell *et al.* with a FT-ICR-MS set-up. For sulfur speciation in petroleum, ESI and APPI sources were compared [133]. APPI produced more ions and was able to ionize samples without requiring chemical derivatization. The elemental composition of the aromatic nitrogen model compounds and the petroleum samples [96] revealed that nitrogen speciation could be obtained by APPI. In-source fragmentation phenomena were avoided by adjusting the instrumental parameters. Finally, Purcell *et al.* observed $[M+H]^+$, $[M-H]^-$ and protonated molecules in the analysis of naphtho[2,3-α]pyrene and crude oil [132], and the latter complex mixture generated more than 12'000 peaks. Contrary to ESI, APPI was able to ionize nonpolar compounds and, therefore, led to nonpolar sulfur speciation in the petrochemical mixtures.

Table 8 : Summary of APPI applications for the analysis of synthetic organics.

analyte	matrix	source	dopant	proportion	polarity	ref
naphtho[2,3-α]pyrene various	standard crude oil	PM	toluene	m.p.	P/N	132
sulfur compounds	petroleum	PM	none	-	P	133
nitrogen aromatics	petroleum	PM	toluene	m.p.	P	96

P : positive polarity
N : negative polarity
P/N : positive and negative polarities
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PM : PhotoMate source

10. Other applications

Low pressure photoionization (LPPI) is another type of source employing photoionization. Because it operates at low pressure (< 1 torr), this technique limits the collisions between ions and molecules that can lead to ionization competition phenomena. However, as mentioned in the introduction, only poor ionization can be achieved at reduced pressure due to the low sample density in the ionization region. The use of a dopant is therefore mandatory. LPPI is usually applied to gas or liquid samples with volatile or semi-volatile analytes [20]. Direct photoionization in LPPI was found to produce $[M]^{\bullet+}$ ions (eq 1, 2), followed by proton transfer from the solvent (eq 8 by replacing the dopant with the solvent) to the analyte, thereby leading to the $[M+H]^+$ ions [134].

PI was also implemented in atmospheric pressure laser ionization (APLI). For some nonpolar or low polar compounds [135], APLI gave a lower LOD compared to APPI due to the higher photon flux than could be obtained with conventional lamps. In fact, APLI photons are typically produced by a KrF excimer laser emitting 5eV photons for efficiently ionizing nonpolar compounds [28]. The main drawbacks to APLI are the large dimension of the source, the tedious maintenance associated with the laser source, and the difficulty in changing the wavelength compared to APPI.

As previously mentioned, APPI has been coupled to IMS [12-15]. Borsdorf *et al.* compared APPI-IMS with APPI-DMS (differential mobility spectrometry) [16;17]. Both techniques provided the same number of ions, indicating that the ionization mechanisms were similar. Besides, the ions' behavior in a radiofrequency, asymmetric electric field was found to be dependent on the molecular structure (the functional groups and the insaturations arrangement). As an example, significant differences in the spectra of aromatic and cyclic hydrocarbons were observed.

Recently, APPI was implemented in miniaturized systems. Haapala *et al.* proposed a microchip nebulizer made of two plates (silicon/glass or glass/glass) for combining APPI/MS with LC or GC capillary [136]. It contained all the classical parts present in a conventional APPI source, namely, a sample inlet channel, auxiliary gas and dopant inlets, vaporizer channels, nozzle and a platinum heater. Luosujärvi *et al.* compared the performance achieved by APCI and APPI microchips for the analysis of polychlorinated biphenyls in a negative-mode GC-API-MS [137]. APPI exhibited a wider linear range and a better repeatability.

Furthermore, the use of an APPI source in GC-MS originally designed for LC-MS was presented in 2007 by McEwen for the analysis of standards [138]. GC-APPI-MS was found to produce similar odd-electron fragment ions as those observed in low energy GC-EI-MS, which are useful for the identification of unknown molecules by comparison with the mass spectra in computer libraries. Ionization with APPI was initiated by the production of the $[M]^{\bullet+}$ ions, which were stable enough to be detected. The $[M]^{\bullet+}$ ions could capture a hydrogen atom from either water vapor, an impurity or a neutral analyte, thereby leading to the $[M+H]^+$ ions. With a homemade lamp model producing 9.8 eV photons, lower fragmentation and more selective ionization were achieved.

Finally, APPI was also used by Haapala *et al.* for the analysis of compounds of various polarities after desorption from different surfaces [102]. With a similar principle to desorption electrospray ionization (DESI), DAPPI required the use of a discharge lamp and a dopant. In this case, the ionization mechanism was found to be a combination of thermal and chemical processes.

11. Conclusion

APPI is the most recent ionization source for the LC - MS coupling. It is a photoinduced chemical ionization process with two major ionization mechanisms in the positive mode, namely, proton and charge transfer. Ionization can only occur for molecules that possess low IE values, while for the other compounds, the use of a dopant is mandatory. The latter can also be employed to enhance the ionization yield of compounds exhibiting low IE values. Acetone and toluene are the most largely reported dopants, but benzene, anisole, hexafluorobenzene and THF have also been used. Toluene was found to produce $[M]^{+\bullet}$ ions and favored the production of $[M+H]^+$ ions by proton transfer. Besides, acetone formed exclusively $[M+H]^+$ ions, but a study of this ionization mechanism is currently not available. In a few cases, the use of a dopant could present a deleterious effect on the ionization. For reversed-phase chromatography and without any dopant, the production of $[M]^{+\bullet}$ ions

by charge transfer was dependent on the mobile phase composition. Methanol was particularly suited with an APPI source. In negative APPI, ionization occurs via six major ionization mechanisms: electron/analyte interaction, charge exchange (electron capture, EC), proton transfer (analyte deprotonation), association/displacement reactions and the newly discovered anion attachment. It generates less background noise than the positive mode and could ionize a broader range of molecules than both negative APCI and ESI. The APPI ionization yield in the positive mode is generally lower than both the ESI and the APCI ionization yields, but, since the solvents used in reversed-phase LC possess IE values that are higher than photon energy, the generated background noise is very low, and high S/N ratios can be obtained. APPI can also be used in combination with other API sources. Because ESI is an orthogonal ionization technique, the dual source ESI/APPI is particularly attractive for the detection of apolar and polar compounds within the same chromatographic run. APPI proved to be an ionization method adapted to a wide panel of compounds and was particularly tailored to separation techniques with low flow rates. Thus, APPI-MS was also found to be particularly adapted to CE due to its high sensitivity at low flow rates and to its compatibility with non volatile buffers. Regarding APPI applications, this ionization technique was found to be particularly useful in the analysis of drugs, human endogenous compounds, lipids, natural compounds, pesticides, synthetic organics and petroleum derivatives. The photoionization mechanisms have been systematically studied and partially elucidated. Nevertheless, knowledge of the APPI mechanisms can still be improved (*i.e.* negative ionization, matrix effects, dopant ionization), and it is assured that future investigations will bring explanations in all of these domains.

Reference List

[1] D.B.Robb, T.R.Covey, A.P.Bruins, Anal. Chem. 72 (2000) 3653.

[2] K.A.Hanold, S.M.Fischer, P.H.Cormia, C.E.Miller, J.A.Syage, Anal. Chem. 76 (2004) 2842.

[3] J.A.Syage, K.A.Hanold, T.C.Lynn, J.A.Horner, R.A.Thakur, J. Chromatogr. A. 1050 (2004) 137.

[4] J.N.Driscoll, F.F.Spaziani, Res. /Dev. 27 (1976) 50.

[5] J.N.Driscoll, Am. Lab. 8 (1976) 71.

[6] J.N.Driscoll. J. Chromatogr. 134 (1977) 49.

[7] M.L.Langhorst, J. Chromatogr. Sci. 19 (1981) 98.

[8] J.T.Schmermund, D.C.Locke, Anal. Lett. 8 (1975) 611.

[9] J.S.M.De Wit, J.W.Jorgenson, J. Chromatogr. 411 (1987) 201.

[10] J.N.Driscoll, D.W.Conron, P.Ferioli, I.S.Krull, K.H.Xie, J. Chromatogr. 302 (1984) 43.

[11] D.C.Locke, B.S.Dhingra, A.D.Baker, Anal. Chem. 54 (1982) 447.

[12] M.A.Baim, R.L.Eatherton, H.H.Hill, Jr., Anal. Chem. 55 (1983) 1761.

[13] G.A.Eiceman, V.J.Vandiver, Anal. Chem. 58 (1986) 2331.

[14] G.A.Eiceman, M.E.Fleischer, C.S.Leasure, Int. J. Environ. Anal. Chem. 28 (1987) 279.

[15] C.S.Leasure, M.E.Fleischer, G.K.Anderson, G.A.Eiceman, Anal. Chem. 58 (1986) 2142.

[16] H.Borsdorf, E.G.Nazarov, R.A.Miller, Anal. Chim. Acta. 575 (2006) 76.

[17] H.Borsdorf, E.G.Nazarov, R.A.Miller, Talanta. 71 (2007) 1804.

[18] E.G.Nazarov, R.A.Miller, G.A.Eiceman, J.A.Stone, Anal. Chem. 78 (2006) 4553.

[19] I.A.Revel'skii, Y.Yashin, V.N.Voznesenskii, V.K.Kurochkin, and R.G.Kostyanovskii. Mass spectrometric analysis of gas mixture. USSR. 83-3546684[1159412]. 24-1-1983. SU.

[20] J.A.Syage, M.D.Evans, K.A.Hanold, Am. Lab. 32 (2000) 24.

[21] E.Marotta, R.Seraglia, F.Fabris, P.Traldi, Int. J. Mass Spectrom. 228 (2003) 841.

[22] A.Raffaelli, A.Saba, Mass Spectrometry Reviews. 22 (2003) 318.

[23] S.J.Bos, S.M.Leeuwen, U.Karst, Anal. Bioanal. Chem. 384 (2006) 85.

[24] T.J.Kauppila, R.Kostiainen, A.P.Bruins, Rapid Commun. Mass Spectrom. 18 (2004) 808.

[25] D.I.Carroll, I.Dzidic, E.C.Horning, R.N.Stillwell, Appl. Spectrosc. Rev. 17 (1981) 337.

[26] T.J.Kauppila, A.P.Bruins, R.Kostiainen, J. Am. Soc. Mass Spectrom. 16 (2005) 1399.

[27] D.B.Robb, M.W.Blades, J. Am. Soc. Mass Spectrom. 17 (2006) 130.

[28] L.C.Short, S.S.Cai, J.A.Syage, J. Am. Soc. Mass Spectrom. 18 (2007) 589.

[29] S.Tomoda, K.Kimura, Bull. Chem. Soc. Jpn. 56 (1983) 1768.

[30] R.A.Provencal, J.B.Paul, K.Roth, C.Chapo, R.N.Casaes, R.J.Saykally, G.S.Tschumper, H.F.Schaefer, III, J. Chem. Phys. 110 (1999) 4258.

[31] T.J.Kauppila, T.Kuuranne, E.C.Meurer, M.N.Eberlin, T.Kotiaho, R.Kostiainen, Anal. Chem. 74 (2002) 5470.

[32] E.P.Grimsrud, P.Kebarle, J. Am. Chem. Soc. 95 (1973) 7939.

[33] M.Meot-Ner, J. Am. Chem. Soc. 108 (1986) 6189.

[34] E.Marotta, P.Traldi, Rapid Commun. Mass Spectrom. 17 (2003) 2846.

[35] A.Giuliani, D.Debois, O.Laprevote, Eur. J. Mass Spectrom. 12 (2006) 189.

[36] J.-P.Rauha, H.Vuorela, R.Kostiainen, J. Mass Spectrom. 36 (2001) 1269.
[37] S.N.Ketkar, J.G.Dulak, S.Dheandhanoo, W.L.Fite, Anal. Chim. Acta. 245 (1991) 267.
[38] M.Takino, S.Daishima, T.Nakahara, Rapid Commun. Mass Spectrom. 17 (2002) 383.
[39] M.Takino, S.Daishima, N.Taketoshi, J. Chromatogr. A. 1001 (2003) 67.
[40] M.Takino, S.Daishima, T.Nakahara, Rapid Commun. Mass Spectrom. 17 (2003) 1965.
[41] M.Takino, K.Yamaguchi, T.Nakahara, J. Agric. Food Chem. 52 (2004) 727.
[42] A.Leinonen, T.Kuuranne, R.Kostiainen, J. Mass Spectrom. 37 (2002) 693.
[43] R.Mol, G.J.de Jong, G.W.Somsen, Electrophoresis. 26 (2005) 146.
[44] S.B.Turnipseed, J.E.Roybal, W.C.Andersen, L.R.Kuck, Anal. Chim. Acta. 529 (2005) 159.
[45] R.Mol, G.J.de Jong, G.W.Somsen, Anal. Chem. 77 (2005) 5277.
[46] A.Delobel, S.Roy, D.Touboul, K.Gaudin, D.P.Germain, A.Baillet, F.Brion, P.Prognon, P.Chaminade, O.Laprevote, J. Mass Spectrom. 41 (2006) 50.
[47] S.Roy, A.Delobel, K.Gaudin, D.Touboul, D.P.Germain, A.Baillet, P.Prognon, O.Laprevote, P.Chaminade, J. Chromatogr. A. 1117 (2006) 154.
[48] J.B.Jean-Denis, R.Pezet, R.Tabacchi, J. Chromatogr. A. 1112 (2006) 263.
[49] M.Varga, T.Bartok, A.Mesterhazy, J. Chromatogr. A. 1103 (2006) 278.
[50] V.Kertesz, G.J.van Berkel, J. Am. Soc. Mass Spectrom. 13 (2002) 109.
[51] R.Mohamed, Y.A.Hammel, M.H.LeBreton, J.C.Tabet, L.Jullien, P.A.Guy, J. Chromatogr. A. 1160 (2007) 194.
[52] M.Himmelsbach, M.Haunschmidt, W.Buchberger, C.W.Klampfl, J. Chromatogr. A. 1159 (2007) 58.
[53] Y.Hsieh, C.J.G.Duncan, S.Lee, M.Liu, J. Pharm. Biomed. Anal. 44 (2007) 492.
[54] P.Hommerson, A.M.Khan, G.J.de Jong, G.W.Somsen, Electrophoresis. 28 (2007) 1444.
[55] A.Bagag, A.Giuliani, O.Laprevote, Int. J. Mass Spectrom. 264 (2007) 1.
[56] S.S.Cai, K.A.Hanold, J.A.Syage, Anal. Chem. 79 (2007) 2491.
[57] I.Marchi, S.Rudaz, M.Selman, J.L.Veuthey, J. Chromatogr. B. 845 (2007) 244.
[58] S.Grosse, T.Letzel, J. Chromatogr. A. 1139 (2007) 75.
[59] D.Debois, A.Giuliani, O.Laprevote, J. Mass Spectrom. 41 (2006) 1554.
[60] N.Itoh, Y.Aoyagi, T.Yarita, J. Chromatogr. A. 1131 (2006) 285.
[61] L.C.Silva, L.S.O.B.Oliveira, G.D.Mendes, G.Garcia, A.d.S.Pereira, G.De Nucci, J. Chromatogr. B. 832 (2006) 302.
[62] Y.Hsieh, K.Merkle, G.Wang, J.-M.Brisson, A.Korfmacher, Anal. Chem. 75 (2003) 3122.
[63] Y.Hsieh, K.Merkle, G.Wang, Rapid Commun. Mass Spectrom. 17 (2003) 1775.
[64] T.J.Kauppila, T.Kotiaho, R.Kostiainen, A.P.Bruins, J. Am. Soc. Mass Spectrom. 15 (2004) 203.
[65] A.Delobel, F.Halgand, B.Laffranchise-Gosse, H.Snijders, O.Laprévote, Anal. Chem. 75 (2003) 5961.
[66] H.Keski-Hynnilä, M.Kurkela, E.Elovaara, L.Antonio, J.Magdalou, L.Luukkanen, J.Taskinen, R.Kostiainen, Anal. Chem. 74 (2002) 3449.
[67] C.Yang, J.Henion, J. Chromatogr. A. 970 (2002) 155.

[68] K.S.Hakala, L.Laitinen, A.M.Kaukonen, J.Hirvonen, R.Kostiainen, T.Kotiaho, Anal. Chem. 75 (2003) 5969.

[69] M.J.Greig, B.Bolanos, T.Quenzer, M.R.Bylund Jessica, Rapid Commun. Mass Spectrom. 17 (2003) 2763.

[70] E.R.Trosken, E.Straube, W.K.Lutz, W.Volkel, C.Patten, J. Am. Soc. Mass Spectrom. 15 (2004) 1216.

[71] R.Geyer, A.D.Peacock, D.C.White, C.Lytle, G.J.van Berkel, J. Mass Spectrom. 39 (2004) 922.

[72] H.Moriwaki, M.Ishitake, S.Yoshikawa, H.Miyakoda, J.F.Alary, Anal. Sci. 20 (2004) 375.

[73] G.Wang, Y.Hsieh, W.A.Korfmacher, Anal. Chem. 77 (2005) 541.

[74] E.A.Straube, W.Dekant, W.Voelkel, J. Am. Soc. Mass Spectrom. 15 (2004) 1853.

[75] H.B.Theron, C.Coetzee, F.C.W.Sutherland, J.L.Wiesner, K.J.Swart, J. Chromatogr. B. 813 (2004) 331.

[76] S.M.Van Leeuwen, L.Hendriksen, U.Karst, J. Chromatogr. A. 1058 (2004) 107.

[77] T.J.Kauppila, P.Oestman, S.Marttila, R.A.Ketola, T.Kotiaho, S.Franssila, R.Kostiainen, Anal. Chem. 76 (2004) 6797.

[78] J.Lembcke, U.Ceglarek, G.M.Fiedler, S.Baumann, A.Leichtle, J.Thiery, J. Lipid Res. 46 (2005) 21.

[79] M.M.Kushnir, R.Neilson, W.L.Roberts, A.L.Rockwood, Clinical biochemistry. 37 (2004) 357.

[80] D.B.Robb, M.W.Blades, J. Am. Soc. Mass Spectrom. 16 (2005) 1275.

[81] Y.Cai, D.Kingery, O.McConnell, A.C.Bach, II, Rapid Commun. Mass Spectrom. 19 (2005) 1717.

[82] J.L.Gomez-Ariza, A.Arias-Borrego, T.Garcia-Barrera, Rapid Commun. Mass Spectrom. 20 (2006) 1181.

[83] A.K.Kabler, S.Chen, J. Agric. Food Chem. 54 (2006) 6153.

[84] T.J.Kauppila, T.Nikkola, R.A.Ketola, R.Kostiainen, J. Mass Spectrom. 41 (2006) 781.

[85] J.L.Gomez-Ariza, T.Garcia-Barrera, F.Lorenzo, Anal. Chim. Acta. 570 (2006) 101.

[86] A.Yamamoto, N.Kakutani, K.Yamamoto, T.Kamiura, H.Miyakoda, Environ. Sci. Technol. 40 (2006) 4132.

[87] A.Mueller, M.Mickel, R.Geyer, R.Ringseis, K.Eder, H.Steinhart, J. Chromatogr. B. 837 (2006) 147.

[88] A.V.Talyzin, Y.O.Tsybin, J.M.Purcell, T.M.Schaub, Y.M.Shulga, D.Noreus, T.Sato, A.Dzwilewski, B.Sundqvist, A.G.Marshall, J. Phys. Chem. A. 110 (2006) 8528.

[89] E.R.Troesken, K.Fischer, W.Voelkel, W.K.Lutz, Toxicology. 219 (2006) 33.

[90] A.Dorcier, P.J.Dyson, J.S.McIndoe, Eur. J. Inorg. Chem. (2003) 4294.

[91] A.Munoz-Garcia, J.Ro, J.C.Brown, J.B.Williams, J. Chromatogr. A. 1133 (2006) 58.

[92] M.Papanastasiou, A.W.McMahon, N.S.Allen, A.M.Doyle, B.J.Johnson, K.Keck-Antoine, Polym. Degrad. Stab. 91 (2006) 2675.

[93] J.L.Gomez-Ariza, A.Arias-Borrego, T.Garcia-Barrera, R.Beltran, Talanta. 70 (2006) 859.

[94] L.Song, A.D.Wellman, H.Yao, J.E.Bartmess, J. Am. Soc. Mass Spectrom. 18 (2007) 1789.

[95] J.H.Banoub, B.Benjelloun-Mlayah, F.Ziarelli, N.Joly, M.Delmas, Rapid Commun. Mass Spectrom. 21 (2007) 2867.

[96] J.M.Purcell, R.P.Rodgers, C.L.Hendrickson, A.G.Marshall, J. Am. Soc. Mass Spectrom. 18 (2007) 1265.
[97] Y.S.Ding, D.L.Ashley, C.H.Watson, J. Agric. Food Chem. 55 (2007) 5966.
[98] H.B.Theron, M.J.van der Merwe, K.J.Swart, J.H.van der Westhuizen, Rapid Commun. Mass Spectrom. 21 (2007) 1680.
[99] L.C.Short, K.A.Hanold, S.S.Cai, J.A.Syage, Rapid Commun. Mass Spectrom. 21 (2007) 1561.
[100] L.Song, A.D.Wellman, H.Yao, J.Adcock, Rapid Commun. Mass Spectrom. 21 (2007) 1343.
[101] H.Moriwaki, H.Miyakoda, Rapid Commun. Mass Spectrom. 21 (2007) 1090.
[102] M.Haapala, J.Pol, V.Saarela, V.Arvola, T.Kotiaho, R.A.Ketola, S.Franssila, T.J.Kauppila, R.Kostiainen, Anal. Chem. 79 (2007) 7867.
[103] A.Bacaloni, C.Cavaliere, A.Faberi, P.Foglia, A.Marino, R.Samperi, A.Lagana, Talanta. 72 (2007) 419.
[104] D.B.Robb, M.W.Blades, Anal. Chem. 78 (2006) 8162.
[105] M.Tubaro, E.Marotta, R.Seraglia, P.Traldi, Rapid Commun. Mass Spectrom. 17 (2003) 2423.
[106] E.Basso, E.Marotta, R.Seraglia, M.Tubaro, P.Traldi, J. Mass Spectrom. 38 (2003) 1113.
[107] I.Dzidic, D.I.Carroll, R.N.Stillwell, E.C.Horning, J. Am. Chem. Soc. 96 (1974) 5258.
[108] F.G.Kitson, B.S.Larsen, C.N.McEwen, Editors. Gas Chromatography and Mass Spectrometry, Academic Press, San Diego, 1996.
[109] C.Cavaliere, P.Foglia, E.Pastorini, R.Samperi, A.Lagana, J. Chromatogr. A. 1101 (2006) 69.
[110] S.S.Cai, J.A.Syage, Anal. Chem. 78 (2006) 1191.
[111] L.C.Short, J.A.Syage, Rapid Commun. Mass Spectrom. 22 (2008) 541.
[112] R.Bonfiglio, R.C.King, T.V.Olah, K.Merkle, Rapid Commun. Mass Spectrom. 13 (1999) 1175.
[113] J.Schappler, D.Guillarme, J.Prat, J.L.Veuthey, S.Rudaz, Electrophoresis. 28 (2007) 3078.
[114] S.L.Nilsson, C.Andersson, P.J.R.Sjoberg, D.Bylund, P.Petersson, M.Jornten-Karlsson, K.E.Markides, Rapid Commun. Mass Spectrom. 17 (2003) 2267.
[115] P.Hommerson, A.M.Khan, T.Bristow, W.Niessen, G.J.de Jong, G.W.Somsen, Anal. Chem. 79 (2007) 5351.
[116] M.Himmelsbach, M.Haunschmidt, W.Buchberger, C.W.Klampfl, Anal. Chem. 79 (2007) 1564.
[117] J.Schappler, D.Guillarme, S.Rudaz, J.L.Veuthey, Electrophoresis. 29 (2008) 11.
[118] J.A.Starkey, Y.Mechref, J.Muzikar, W.J.McBride, M.V.Novotny, Anal. Chem. 78 (2006) 3342.
[119] J.Martens-Lobenhoffer, I.Reiche, U.Troeger, K.Moenkemueller, P.Malfertheiner, S.M.Bode-Boeger, J. Chromatogr. B. 857 (2007) 301.
[120] A.d.S.Pereira, L.S.O.B.Oliveira, G.D.Mendes, J.J.Gabbai, G.De Nucci, J. Chromatogr. B. 828 (2005) 27.
[121] S.S.Cai, J.A.Syage, J. Chromatogr. A. 1110 (2006) 15.
[122] A.Munoz-Garcia, J.Ro, J.C.Brown, J.B.Williams, J. Exp. Biol. 211 (2008) 447.
[123] J.Cao, S.J.Murch, R.O'Brien, P.K.Saxena, J. Chromatogr. A. 1134 (2006) 333.
[124] O.Pardo, V.Yusa, N.Leon, A.Pastor, J. Chromatogr. A. 1154 (2007) 287.
[125] L.Tonidandel, E.Ragazzi, G.Roghi, P.Traldi, Rapid Commun. Mass Spectrom. 22 (2008) 630.
[126] A.Riu, D.Zalko, L.Debrauwer, Rapid Commun. Mass Spectrom. 20 (2006) 2133.

[127] J.Zheng, S.A.Shamsi, Anal. Chem. 78 (2006) 6921.

[128] C.Crescenzi, J.Albinana, H.Carlsson, E.Holmgren, R.Batlle, J. Chromatogr. A. 1153 (2007) 186.

[129] J.W.Davis, S.J.Gonsior, D.A.Markham, U.Friederich, R.W.Hunziker, J.M.Ariano, Environ. Sci. Technol. 40 (2006) 5395.

[130] L.Debrauwer, A.Riu, M.Jouahri, E.Rathahao, I.Jouanin, J.P.Antignac, R.Cariou, B.Le Bizec, D.Zalko, J. Chromatogr. A. 1082 (2005) 98.

[131] A.Riu, J.Cravedi, L.Debrauwer, A.Garcia, C.Canlet, I.Jouanin, D.Zalko, Environ. Int. 34 (2008) 318.

[132] J.M.Purcell, C.L.Hendrickson, R.P.Rodgers, A.G.Marshall, Anal. Chem. 78 (2006) 5906.

[133] J.M.Purcell, P.Juyal, D.G.Kim, R.P.Rodgers, C.L.Hendrickson, A.G.Marshall, Energy Fuels. 21 (2007) 2869.

[134] J.A.Syage, J. Am. Soc. Mass Spectrom. 15 (2004) 1521.

[135] M.Constapel, M.Schellentraeger, O.J.Schmitz, S.Gaeb, K.J.Brockmann, R.Giese, T.Benter, Rapid Commun. Mass Spectrom. 19 (2005) 326.

[136] M.Haapala, L.Luosujaervi, V.Saarela, T.Kotiaho, R.A.Ketola, S.Franssila, R.Kostiainen, Anal. Chem. 79 (2007) 4994.

[137] L.Luosujarvi, M.M.Karikko, M.Haapala, V.Saarela, S.Huhtala, S.Franssila, R.Kostiainen, T.Kotiaho, T.J.Kauppila, Rapid Commun. Mass Spectrom. 22 (2008) 425.

[138] C.N.McEwen, Int. J. Mass Spectrom. 259 (2007) 57.

[139] S.T.Tsai, J.C.Jiang, Y.T.Lee, A.H.Kung, S.H.Lin, C.K.Ni, J. Chem. Phys. 111 (1999) 3434.

Article II

Evaluation of the influence of protein precipitation prior to on-line SPE-LC-API/MS procedures using multivariate data analysis [1]

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[1] This paper was presented at the 10th International Symposium on Biochromatography, Lille, France, 26-28 April 2006

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Journal of Chromatography B, 845 (2007) 244–252

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 photo-ionization (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 thirty years ago as a detection method (PID) in gas chromatography [7-12] and coupling LC to PI was performed by Locke [13], and later by Driscoll [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 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) (Figure 1).

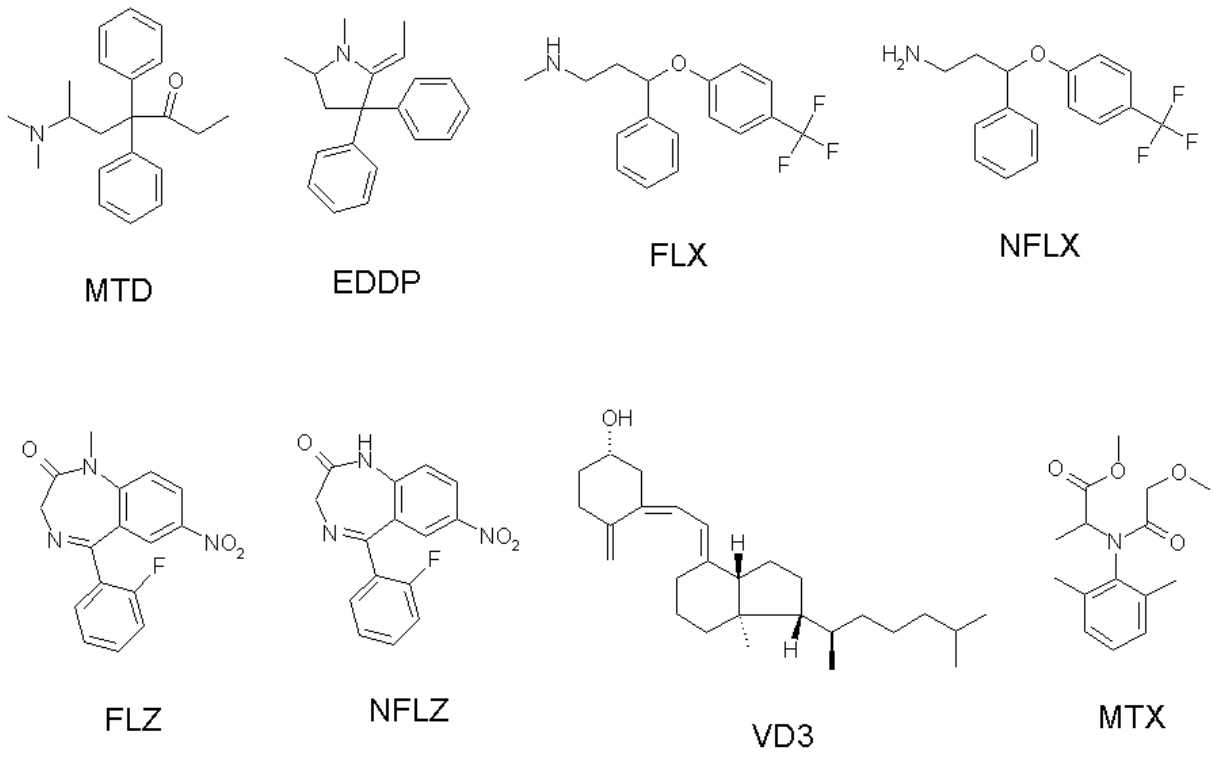

Figure 1 : Chemical structures of model compounds.

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 Figure 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 $\mu g \cdot mL^{-1}$, each. Stock solution of VD3 was prepared in methanol at a concentration of 1000 $\mu g \cdot mL^{-1}$. For the optimization of the ion source parameters, standard solutions at a concentration of 10 $\mu g \cdot mL^{-1}$ were prepared by dilution of stock solutions in the mobile phase. For post-column infusion, a solution containing the eight drugs at 2 $\mu g \cdot mL^{-1}$ was prepared by dilution of stock solutions in the mobile phase. Blank plasma was stored at -22℃ and then defrosted at room temperature for one hour.

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).

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 (ºC)	300	350	300
capillary voltage (V)	3000	3500	2000
vaporizer temperature (ºC)	-	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 Sample handling

2.3.1 Protein precipitation with acetonitrile [50]

500 µL of blank plasma was added to 1000 µL of acetonitrile, vortex mixed and centrifuged for 5 minutes at 6000 *x g*. The supernatant was collected and transferred into a vial.

2.3.2 Protein precipitation with perchloric acid / acetonitrile

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

2.3.3 Dilution with water (direct injection)

750 µL of blank plasma was added to 750 µL of water, vortex mixed and centrifuged for 5 minutes at 6000 *x 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 Figure 2 as already reported elsewhere [32]. A chromatographic Purospher STAR RP-18e column (55 x 2.0 mm I.D., d_p 3 µm) from Merck (Darmstad, Germany) was used for chromatographic separations. Selected extraction supports, coupled on-line with the analytical column, were : an Oasis HLB (20 x 2.1 mm

I.D., d_p 25 µm) from Waters Corporation (MA, USA), a LiChrospher RP-4 ADS (25 x 2.0 mm I.D., d_p 25 µm) from Merck (Darmstadt, Germany), a Cyclone Turboflow HTLC (50 x 1.0 mm I.D., d_p 50 µm) from Cohesive Technologies (MA, USA) and a Chromolith Flash (25 x 4.6 mm I.D.) from Merck.

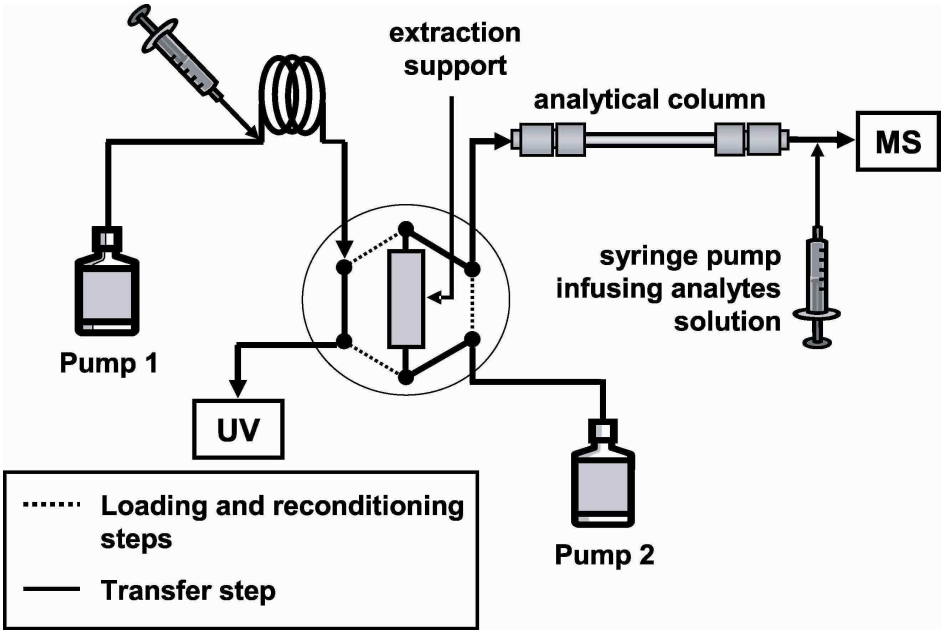

Figure 2 : Column-switching configuration with post-column infusion system.

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}.

50 µL 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.

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

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.

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 based on 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 Figure 3.

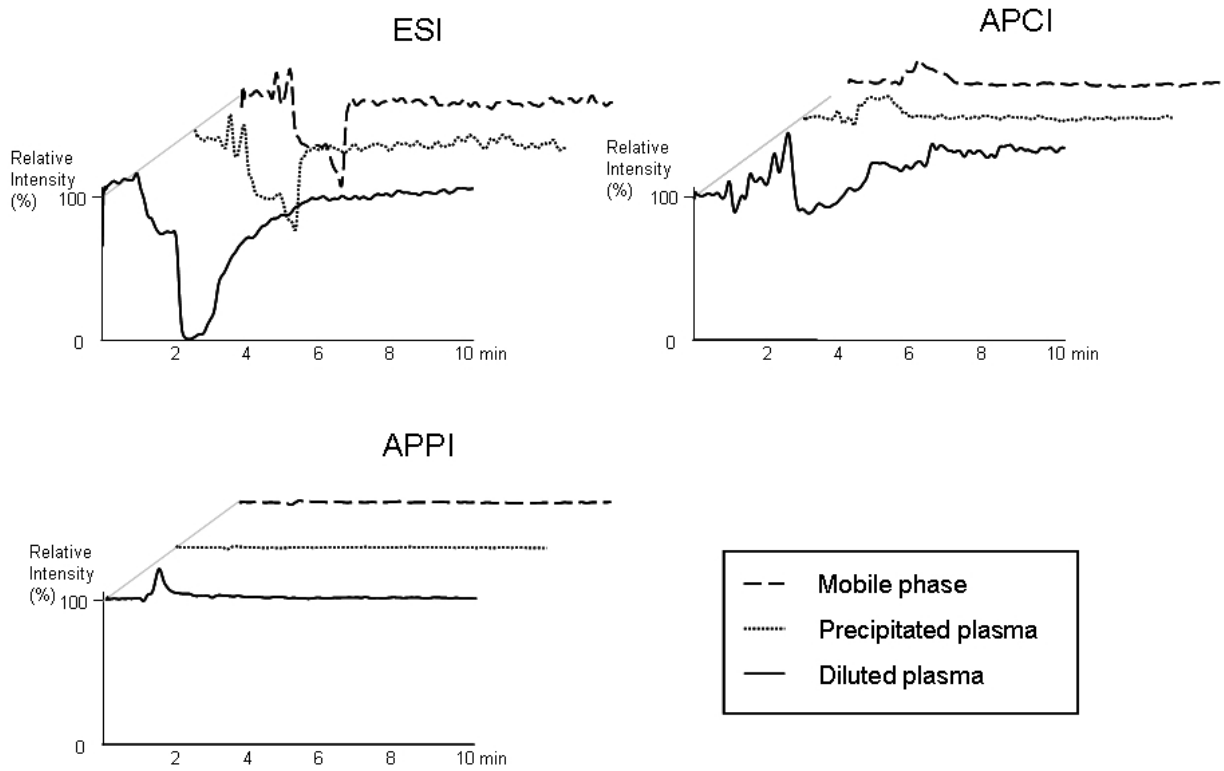

Figure 3 : TIC profiles of samples injections on the Chromolith Flash support.

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 (Figure 4).

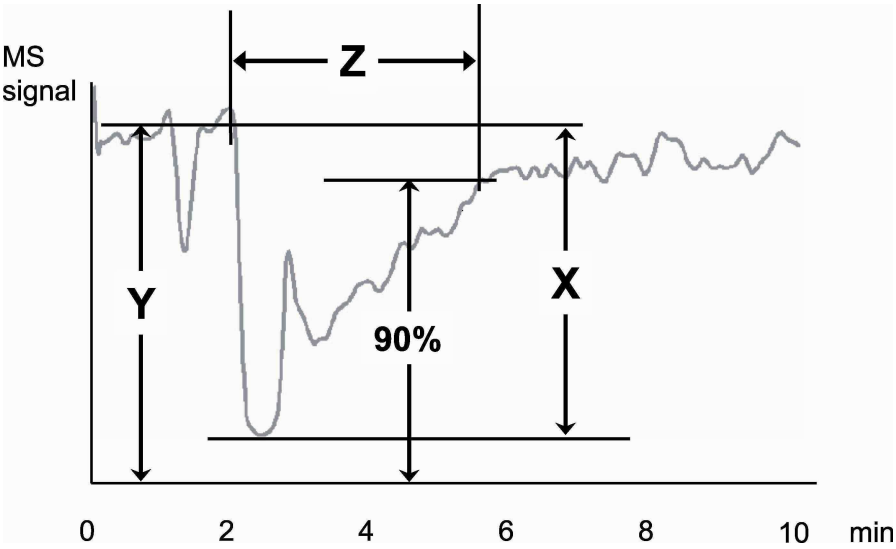

Figure 4 : Maximal signal alteration value (**X**), baseline value (**Y**) and matrix effect time window (**Z**).

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 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 x 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 minutes in ESI and about 6 minutes 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 mL·min^{-1} or at 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 x 2.1 mm I.D. was used instead of a 50 x 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.

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 $mL \cdot min^{-1}$ (ADSs), LiChrospher RP-4 ADS used at 4.0 $mL \cdot min^{-1}$ (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
	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

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 7 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 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.

Figure 5 presents the concentration measured based on 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 4 times less sensitive, followed by APPI (ca. 10 times less sensitive). Therefore the choice of the ionization source must be made considering two criteria : matrix effect influence and detection sensitivity.

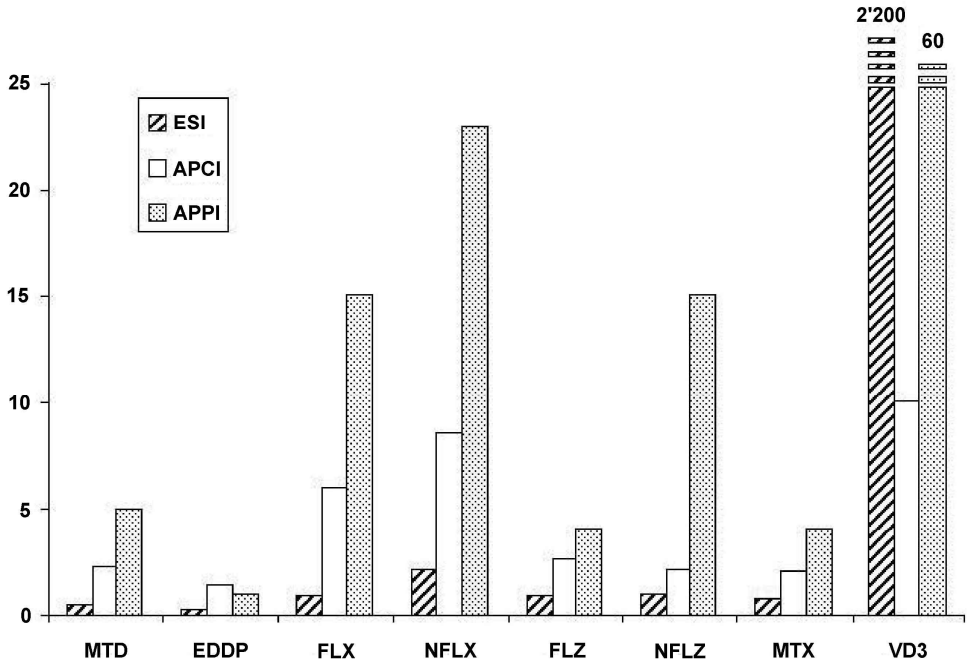

Figure 5 : Analytes sensitivity (S/N = 10) with the tested ionization sources.

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 (Figure 6).

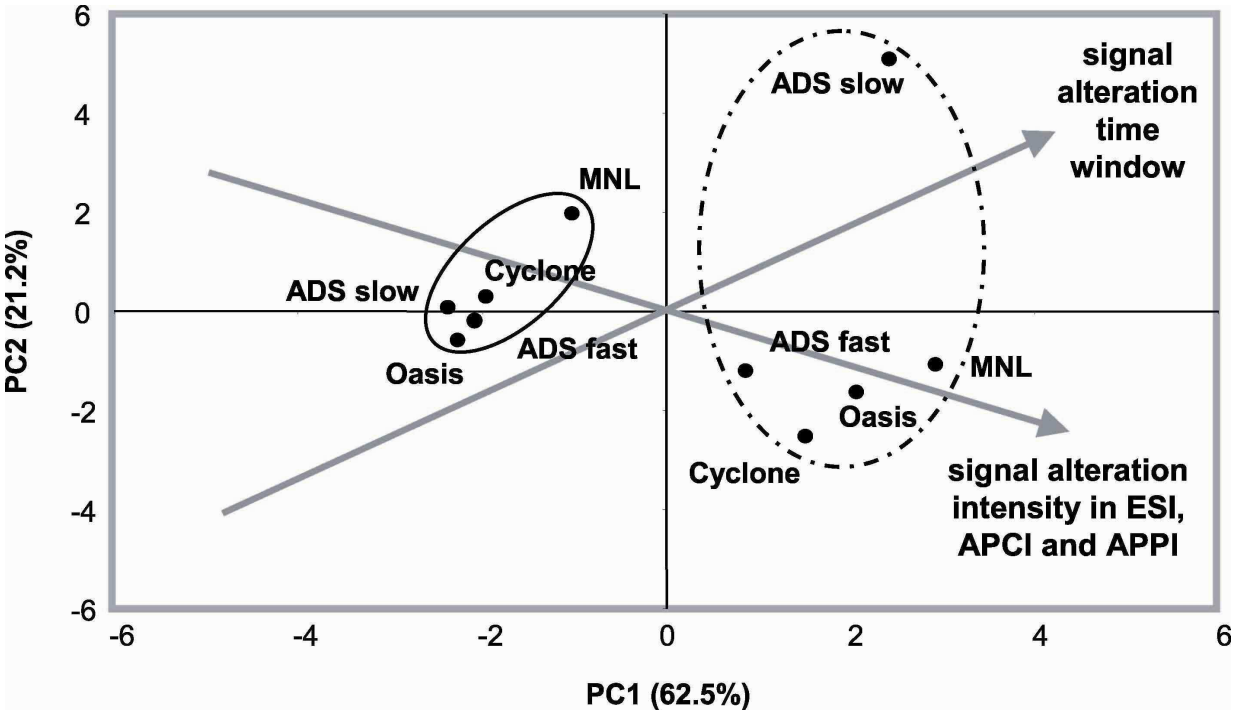

Figure 6 : Global Principal Components Analysis. Dot line : diluted plasma. Continuous line : precipitated plasma.

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 Figure 6, PCA demonstrated a good clusterisation 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 Figure 6, LiChrospher RP-4 ADS used at 0.8 $mL \cdot min^{-1}$ (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 (IS) 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. Based on 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.

Reference List

[1] J.Abian, J. Mass Spectrom. 34 (1999) 157.

[2] C.K.Lim, G.Lord, Biol. Pharm. Bull. 25 (2002) 547.

[3] P.Marquet, G.Lachatre, J. Chromatogr. B. 733 (1999) 93.

[4] H.H.Maurer, J. Chromatogr. B. 713 (1998) 3.

[5] W.M.A.Niessen, A.P.Tinke, J. Chromatogr. A. 703 (1995) 37.

[6] W.M.A.Niessen, J. Chromatogr. A. 794 (1998) 407.

[7] J.N.Driscoll, J.B.Clarici, Chromatographia. 9 (1976) 567.

[8] J.N.Driscoll, F.F.Spaziani, Research/Development. 27 (1976) 50.

[9] J.N.Driscoll. J. Chromatogr. A. 134 (1976) 49.

[10] J.N.Driscoll, F.F.Spaziani, Chemie Magazine. 2 (1976) 105.

[11] J.N.Driscoll, NBS Special Publication. 464 (1977) 415.

[12] J.N.Driscoll, American Laboratory. 8 (1976) 71.

[13] D.C.Locke, B.S.Dhingra, A.D.Baker, Anal. Chem. 54 (1982) 447.

[14] J.N.Driscoll, D.W.Conron, P.Ferioli, I.S.Krull, K.H.Xie, J. Chromatogr. A. 302 (1984) 43.

[15] I.A.Revel'skii, Y.Yashin, V.N.Voznesenskii, V.K.Kurochkin, R.G.Kostyanovskii, Izvestiya Akademii Nauk SSSR, Seriya Khimicheskaya. (1986) 1987.

[16] D.B.Robb, T.R.Covey, A.P.Bruins, Anal. Chem. 72 (2000) 3653.

[17] J.A.Syage, M.D.Evans, K.A.Hanold, American Laboratory. 32 (2000) 24.

[18] H.Mei, Y.Hsieh, C.Nardo, X.Xu, S.Wang, K.Ng, W.A.Korfmacher, Rapid Commun. Mass Spectrom. 17 (2003) 97.

[19] R.Bonfiglio, R.C.King, T.V.Olah, K.Merkle, Rapid Commun. Mass Spectrom. 13 (1999) 1175.

[20] D.L.Buhrman, P.I.Price, P.J.Rudewicz, J. Am. Soc. Mass Spectrom. 7 (1996) 1099.

[21] B.K.Choi, D.M.Hercules, A.I.Gusev, J. Chromatogr. A. 907 (2001) 337.

[22] R.Dams, M.A.Huestis, W.E.Lambert, C.M.Murphy, J. Am. Soc. Mass Spectrom. 14 (2003) 1290.

[23] C.G.Enke, Anal. Chem. 69 (1997) 4885.

[24] J.Eshraghi, S.K.Chowdhury, Anal. Chem. 65 (1993) 3528.

[25] E.T.Gangl, M.Annan, N.Spooner, P.Vouros, Anal. Chem. 73 (2001) 5635.

[26] Y.Hsieh, M.Chintala, H.Mei, J.Agans, J.M.Brisson, K.Ng, W.A.Korfmacher, Rapid Commun. Mass Spectrom. 15 (2001) 2481.

[27] C.R.Mallet, Z.Lu, J.R.Mazzeo, Rapid Commun. Mass Spectrom. 18 (2004) 49.

[28] R.Pascoe, J.P.Foley, A.I.Gusev, Anal. Chem. 73 (2001) 6014.

[29] J.Schuhmacher, D.Zimmer, F.Tesche, V.Pickard, Rapid Commun. Mass Spectrom. 17 (2003) 1950.

[30] L.E.Sojo, G.Lum, P.Chee, Analyst. 128 (2003) 51.

[31] S.Souverain, S.Rudaz, J.-L.Veuthey, J. Pharm. Biomed. Anal. 35 (2004) 913.

[32] S.Souverain, S.Rudaz, J.L.Veuthey, J. Chromatogr. A. 1058 (2004) 61.

[33] P.R.Tiller, L.A.Romanyshyn, Rapid Commun. Mass Spectrom. 16 (2001) 92.

[34] M.W.J.van Hout, H.A.G.Niederlander, R.A.de Zeeuw, G.J.de Jong, Rapid Commun. Mass Spectrom. 17 (2003) 245.
[35] C.Yang, J.Henion, J. Chromatogr. A. 970 (2002) 155.
[36] G.Wang, Y.Hsieh, W.A.Korfmacher, Anal. Chem. 77 (2005) 541.
[37] S.B.Turnipseed, J.E.Roybal, W.C.Andersen, L.R.Kuck, Anal. Chim. Acta. 529 (2005) 159.
[38] M.Takino, S.Daishima, T.Nakahara, Rapid Commun. Mass Spectrom. 17 (2003) 1965.
[39] Y.Hsieh, K.Merkle, G.Wang, J.-M.Brisson, A.Korfmacher, Anal. Chem. 75 (2003) 3122.
[40] Y.Hsieh, K.Merkle, G.Wang, Rapid Commun. Mass Spectrom. 17 (2003) 1775.
[41] K.A.Hanold, S.M.Fischer, P.H.Cormia, C.E.Miller, J.A.Syage, Anal. Chem. 76 (2004) 2842.
[42] B.K.Choi, D.M.Hercules, A.I.Gusev, Fresenius J. Anal. Chem. 369 (2001) 370.
[43] Y.Hsieh, G.Wang, Y.Wang, S.Chackalamannil, J.Brisson, K.Ng, W.A.Korfmacher, Rapid Commun. Mass Spectrom. 16 (2002) 944.
[44] C.Muller, P.Schafer, M.Stortzel, S.Vogt, W.Weinmann, J. Chromatogr. B. 773 (2002) 47.
[45] M.D.Nelson, J.W.Dolan, LCGC North Am. 20 (2002) 24.
[46] W.Z.Shou, W.Naidong, Rapid Commun. Mass Spectrom. 17 (2003) 589.
[47] T.L.Constantopoulos, G.S.Jackson, C.G.Enke, J. Am. Soc. Mass Spectrom. 10 (1999) 625.
[48] B.K.Matuszewski, M.L.Constanzer, C.M.Chavez-Eng, Anal. Chem. 70 (1998) 882.
[49] J.Blanchard, J. Chromatogr. A. 226 (1981) 455.
[50] C.Polson, P.Sarkar, B.Incledon, V.Raguvaran, R.Grant, J. Chromatogr. B. 785 (2003) 263.
[51] D.A.Wells. High Throughput Bioanalytical Sample Preparation : Methods and Automation Strategies. Elsevier, New York, 2003.
[52] R.A.Biddlecombe, S.Pleasance, J. Chromatogr. B. 734 (1999) 257.
[53] N.Brignol, L.M.McMahon, S.Luo, F.L.S.Tse, Rapid Commun. Mass Spectrom. 15 (2001) 898.
[54] Z.Shen, S.Wang, R.Bakhtiar, Rapid Commun. Mass Spectrom. 16 (2002) 332.
[55] S.Souverain, S.Rudaz, J.-L.Veuthey, G. I. T. Laboratory Journal. 02 (2003) 80.
[56] J.-L.Veuthey, S.Souverain, S.Rudaz, Ther. Drug Monit. 26 (2004) 161.
[57] S.Souverain, S.Rudaz, J.-L.Veuthey, J. Chromatogr. B. 801 (2004) 141.
[58] C.Schafer, D.Lubda, J. Chromatogr. A. 909 (2001) 73.

Article III

Sample preparation development and matrix effects evaluation for multianalyte determination in urine

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Journal of Pharmaceutical and Biomedical Analysis (*In press*, doi:10.1016/j.jpba.2008.11.040*)*

ABSTRACT

The development of a generic analytical method remains difficult when a high number of compounds has to be simultaneously considered. This study proposes an innovative strategy for the development of a solid phase extraction (SPE) procedure before liquid chromatography-mass spectrometry analysis of 34 diuretics and beta-blockers in urine samples. These compounds have been selected since they are often encountered in anti-doping control. The principle is based on the selection of representative analytes during SPE protocol optimization, allowing a drastic reduction of generated data and development time. To select the representative compounds, all substances were classified based on their SPE behavior with a generic method and groups were formed with the help of a chemometric tool, namely hierarchical cluster analysis (HCA). One representative analyte per group was selected and used for subsequent SPE method development. Once the SPE method was developed, compounds were analyzed by LC-MS and matrix effects were evaluated to determine the influence of the matrix on the SPE process and MS signal alteration due to endogenous compounds. As a result, matrix effects evaluation must be performed on all analytes ; representative compounds previously selected for SPE development were unable to predict matrix effects.

Keywords : solid phase extraction, SPE, fast SPE development, representative compounds, hierarchical cluster analysis, HCA

1. Introduction

The analysis of a large number of compounds in biological matrices (e.g. urine, blood) is required in various domains such as therapeutic drug monitoring (TDM), doping control, forensic sciences, and toxicology. From a practical point of view, the use of a reduced number of generic methods, allowing the simultaneous determination of different substances, is generally preferred in routine analyses. When handling biological samples for liquid-chromatography (LC) which remains the method of choice for this kind of application, a sample preparation is mandatory to limit column clogging, presence of co-eluting substances as well as matrix effects frequently encountered with mass spectrometric detection (MS) [1-4]. In this regard, solid phase extraction (SPE) is often chosen, allowing good sample clean-up. The use of on-line SPE in the column-switching mode is particularly adapted to TDM [5], but requires a dedicated instrument. On the other hand, the off-line SPE on multi-well plates is qualified for multianalyte procedures. Since each sample is independently extracted, this format is compatible with different separation techniques working with various mechanisms (reversed or normal phase, ion exchange, etc), reduces contamination risk, which presents a great advantage in analyses where a legal aspect has to be taken into account, and does not require a particular technical skill [6].

The analysis of a large variety of substances can induce a tedious and time consuming method development. To the best of our knowledge, only a small number of studies have reported solutions for time reduction in the development of a generic sample preparation procedure. A solution was the use of an apparatus capable of extracting a large number of samples (i.e. multi-well plates, on-line extraction supports, *etc*.) [7]. In this approach, all analytes of interest are evaluated, inducing a tedious task and generating large amounts of data. Additionally, the use of a chemometric tool was proposed to determine the principal interaction effects of the extraction conditions, leading to a reduced number of experiments for method development [8]. The last approach mentioned in the literature was based on the reduction of used analytes, drastically lowering time and the number of procedures [9]. However, no rigorous compound selection has been proposed, leading to a sample preparation that is not necessarily focused on the full set of compounds.

Moreover, it is well known that the sample preparation must reduce matrix effects on subsequent LC-MS analysis to obtain repeatable and reliable results. A characterization and reduction of these effects must be operated as already described in several publications [10-19]. The method proposed by Matuszewski *et al.* [18], to identify matrix effects was considered in this study to determine their origin and overcome the problem by the use of an internal standard or a modification of the sample preparation prior to validation.

The aim of this paper is to propose a simple and low-cost method to reduce the time required for developing a sample preparation in the case of multianalyte analysis by an appropriate selection of representative analytes. Moreover, matrix effects after the optimized sample preparation are evaluated *via* a method analogous to the one previously mentioned [18], and a classification of the different types of matrix effects is proposed.

2. Experimental

2.1 Chemicals

Acebutolol, acetazolamide, atenolol, bendroflumethiazide, betamethasone, bumetanide, chlorothiazide, chlorthalidone, clopamide, dexamethasone, ethacrynic acid, furosemide, hydrochlorothiazide, indapamide, metolazone, metoprolol, nadolol, probenecid, sotalol, strychnine, formic acid and 37% hydrochloric acid solutions were purchased from Sigma-Aldrich (Buchs, Switzerland). Adrafinil, canrenone, celiprolol, dichlorphenamide, esmolol, gestrinone, piretanide, torasemide and xipamide were kindly provided by the Laboratoire d'Analyse du Dopage (Epalinges, Switzerland). Benzoylecgonine was purchased from Cerilliant (Austin, TX, USA), carteolol was extracted from Arteoptic® tablets (Novopharma, Cham, Switzerland) and finasteride was obtained from Propecia® tablets (MSD, Glattbrugg, Switzerland). Metipranolol was present in Betanol® eye-drops (Europhta, Monaco), and modafinil was extracted from Modasomil® 100 tablets (Cephalon, Martinsried, Germany). A list of pKa, logD and logP values for each compound is reported in Table 1. Acetonitrile (ACN) and methanol (MeOH) were purchased from Panreac (Barcelona, Spain) and water was provided by a Milli-Q Gradient A10 water purifier system from Millipore (Bedford, MA, USA). The ammonia solution (25%) was purchased from Fluka (Buchs, Switzerland). All chemicals were of the highest purity grade commercially available, and all reagents used were of HPLC grade.
Stock solutions of all compounds at 1000 $\mu g \cdot mL^{-1}$ were individually prepared in a mixture of water - ACN (50:50, v/v). Ammonium buffer (50 mM) was prepared every three days by diluting 3.9 mL of ammonia solution (25%) in 500 mL of Milli-Q water. pH was adjusted to pH 10 with a Metrohm 691 pH-meter (Herisau, Switzerland) by adding formic acid drop-by-drop.

Table 1 : List of therapeutic classes, pKa, logD and logP values for all compounds of the set. Values were calculated using Advanced Chemistry Development (ACD/Labs) Software V8.14 for Solaris (© 1994-2006 ACD/Labs)

compound	therapeutic class	pKa acid	pKa basic	logD			logP
				pH1	pH7	pH10	
acebutolol	β-blocker	13.8	9.1	-0.5	0.5	2.5	2.6
acetazolamide	diuretic	7.4	-	-0.3	-0.4	-3.8	-0.2
adrafinil	stimulant	8.2	-	1.0	1.0	-0.8	1.0
atenolol	β-blocker	13.9	9.2	-3.0	-2.0	0.0	0.1
bendroflumethiazide	diuretic	8.6	-	2.0	2.0	-0.1	2.0
benzoylecgonine	narcotic	3.5	10.8	-0.4	0.2	0.2	2.7
betamethasone	corticosteroid	12.1	-	2.1	2.1	2.2	2.0
bumetanide	diuretic	3.2	4.5	0.5	-0.3	-1.5	2.7
canrenone	diuretic	-	-	3.0	3.0	3.0	2.9
carteolol	β-blocker	13.8	9.1	-1.4	-0.4	1.6	1.6
celiprolol	β-blocker	13.8	9.1	-0.3	0.8	2.8	2.8
chlorothiazide	diuretic	9.2	-	-0.2	-0.2	-1.1	-0.1
chlorthalidone	diuretic	9.6	-	-0.7	-0.7	-1.4	-0.7
clopamide	diuretic	9.4	4.1	-1.2	1.6	0.9	1.5
dexamethasone	corticosteroid	12.1	-	1.9	1.9	1.9	1.8
dichlorphenamide	diuretic	9.0	-	0.9	0.9	-0.9	0.9
esmolol	β-blocker	13.9	9.2	-1.2	-0.2	1.9	1.9
ethacrynic acid	diuretic	2.8	-	3.4	-0.5	-0.7	3.3
finasteride	diuretic	-	-	3.2	3.2	3.2	3.2
furosemide	diuretic	3.0	-	2.9	-0.8	-1.5	2.9
gestrinone	anabolic agent	-	-	3.3	3.3	3.3	3.3
hydrochlorothiazide	diuretic	9.0	-	-0.1	-0.1	-1.8	-0.1
indapamide	diuretic	9.4	-	2.1	2.1	1.2	2.1
metipranolol	β-blocker	13.9	9.2	-0.4	0.5	2.6	2.6
metolazone	diuretic	10.0	-	3.2	3.2	2.9	3.1
metoprolol	β-blocker	13.9	9.2	-1.3	-0.3	1.7	1.7
modafinil	stimulant	-	-	1.4	1.4	1.4	1.4
nadolol	β-blocker	13.9	9.2	-1.8	-0.8	1.2	1.2
piretanide	diuretic	10.2	4.3	-0.0	-1.2	-2.4	1.8
probenecid	diuretic	3.7	-	3.3	0.1	-0.8	3.3
sotalol	β-blocker	9.6	9.2	-2.8	-1.8	-0.3	0.3
strychnine	stimulant	-	8.3	-1.4	0.3	1.6	1.6
torasemide	diuretic	3.1	4.8	0.7	0.5	-0.9	3.1
xipamide	diuretic	5.1	-	4.0	2.1	0.5	4.0

2.2 SPE

2.2.1 Generic protocol

All sample extractions were performed on a 10 mg 2 mL Waters Oasis Sorbent Selection Plate (comprising Oasis MCX, Oasis MAX, Oasis WCX and Oasis WAX sorbents [20]) and on an Oasis

HLB 10 mg 2 mL plate using a Waters SPE manifold and a Gast DOA-P504-BN pump (Benton Harbor, MI, USA) kindly loaned by Waters. The generic Oasis 2x4 Method was applied to extract standard solutions with Oasis MCX, WAX, MAX, and WCX. Sorbents were conditioned with 500 µL of MeOH and equilibrated with 500 µL of water. One thousand and five hundred microliters of sample was loaded on each type of sorbent (MCX, MAX, WCX, and WAX). Washing was performed with 900 µL of 2% HCOOH for MCX, and WAX and 900 µL of NH_4OH in water (5:95, v/v) for MAX and WCX. The first elution was assessed with 900 µL of MeOH, and the second elution was performed with 900 µL of NH_4OH in MeOH (5:95, v/v) for MCX and WAX and with 900 µL of HCOOH in MeOH (2:98, v/v) for MAX and WCX. Extraction on Oasis HLB was evaluated with loading in acidic or neutral media. When loading in acidic conditions, the sorbent was conditioned with 500 µL of MeOH and equilibrated with 500 µL of HCl (120 mM). One thousand and five hundred microliters of acidified sample (120 mM HCl) was loaded. Washing was performed with 900 µL of HCOOH in water (2:98, v/v) and elution with 900 µL of MeOH. When loading in neutral conditions, the sorbent was conditioned with 500 µL of MeOH and equilibrated with 500 µL of water. A volume of 1500 µL of sample was loaded. Washing was performed with 900 µL of water and elution with 900 µL of MeOH. All fractions were evaporated to dryness from the 96-well collection plate using a Univapo 150 ECH (UniEquip, Martinsried, Germany) vacuum concentrator centrifuges set at 40°C. Samples were reconstituted in 200 µL of water. Collection plates were directly used as injection vials for the UPLC.

2.2.2 Optimized SPE protocol (Oasis MCX)

The sample (urine spiked with all tested substances at 100 ppb each) was first centrifuged at 10'000 g for 10 minutes, and 750 µL of HCl (240 mM) was added to 750 µL of the collected supernatant. The sorbent was conditioned with 500 µL of MeOH and equilibrated with 500 µL of HCl (120 mM). A volume of 1000 µL of the acidified sample was loaded and washed with a mixture of HCl (120 mM) - MeOH (90:10, v/v). First elution was performed with 250 µL of MeOH and the second elution with the same volume of NH_4OH in MeOH (5:95, v/v). Both elutions were evaporated to dryness with the same apparatus as used in paragraph 2.2.1 and reconstituted in 50 µL of a mixture of water - MeOH (50:50, v/v).

2.3 Chromatographic separation

Chromatographic separation was optimized with Osiris (Datalys, Grenoble, France), a HPLC modeling software.

Analyses were performed on a Waters Acquity UPLC system (Milford, MA, USA) equipped with a binary solvent manager, an auto-sampler with a 2 µL injection loop and a stainless steel needle (allowing for injections directly from 96-well plates capped with a silicon sealing cap), and a UV-Vis

programmable detector, including a 500 nL flow cell. The Empower Software was used for instrument control, data acquisition and data handling.
For all separations, a volume of 1 µL was injected on an Acquity BEH Shield RP18, 2.1 x 50 mm, 1.7 µm column used with a gradient of acetonitrile in a ammonium buffer (50 mM, pH 10) from 11.3% to 51.2% in 2.2 min delivered at 900 $\mu L \cdot min^{-1}$. UV detection was operated at 254 nm with a 25 ms time constant and data sampling rate set at 80 Hz.

2.4 Matrix effect evaluation

All experiments for matrix effect evaluation were performed on an Agilent Series 1100 LC system (Agilent Technologies, Waldbronn, Germany) equipped with an autosampler and a binary pump. 5 µL of the sample was injected on a XBridge Shield 100 x 2.1 mm, 3.5 µm analytical column from Waters. The mobile phase (acetate buffer 20 mM pH 5 / ACN 67/33 (v/v)) was delivered in the isocratic mode at 300 $\mu L \cdot min^{-1}$. The LC system was coupled to an Agilent Series 1100 MSD single quadrupole equipped with an orthogonal ESI source. Nitrogen was used as both nebulizing (5 $L \cdot min^{-1}$) and drying gas (250℃). Vaporizer temperature was set at 250℃ , nebulizer pressure at 45 psig and capillary voltage at +2000 V. Detection of protonated analytes was always conducted in the selected ion monitoring (SIM) mode. The Chemstation A.10.03 software (Agilent Technologies) was used for instrument control, data acquisition and data handling.

2.4.1 Neat standards

Five microliters of each stock solution at 10.000 $\mu g \cdot mL^{-1}$ was added to 1000 µL of water. Seven hundred and fifty microliters of HCl (240 mM) was added to 750 µL of the neat standard and vortex mixed. One thousand microliters was finally extracted with the optimized protocol described in section 2.2.2.

2.4.2 Pre-extraction spiked urine

One thousand microliters of blank urine was spiked with 5 µL of each stock solution at 10.000 $\mu g \cdot mL^{-1}$. The sample was then centrifuged at 10'000 g for 10 minutes. Seven hundred and fifty microliters of HCl (240 mM) was added to 750 µL of the collected supernatant and vortex mixed. One thousand microliters was finally extracted with the optimized protocol described in section 2.2.2.

2.4.3 Post-extraction spiked urine

One thousand microliters of blank urine was centrifuged at 10'000 g for 10 minutes. Seven hundred and fifty microliters of HCl (240 mM) was added to 750 µL of the collected supernatant and vortex mixed. One thousand microliters was extracted with the optimized protocol described in section 2.2.2. Finally, the resulting elutions were spiked with 5 µL of a standard solution at 200 $\mu g \cdot mL^{-1}$ of each corresponding analyte.

2.5 Data handling software

Data analysis, including principal component analysis (PCA) and hierarchical cluster analysis (HCA), was performed with the XLStat 7.5.3 (AddinSoft, France) software package. For HCA, a flexible linkage ($\beta = -0.1$) aggregation based on Euclidean distance was selected.

3. Results and discussion

3.1 Compound descriptions

Clinical, forensic, and antidoping laboratories currently employ different screening methods for the analysis of drug substances in urine. In this paper, the determination of a mixture of 34 molecules was studied constituted by 18 diuretics (12 acids, 2 neutrals and 4 bases), 9 β-blockers (ampholytes), 2 corticosteroids (bases), 3 stimulants (1 acid, 1 neutral and 1 basic), 1 narcotic (ampholyte) and 1 anabolic agent (neutral). Thus, acidic compounds and ampholytes represent the majority of studied analytes (Table 1). In regards to polarity, 27 molecules show high logP values ($logP \geq 1$) and 7 exhibit intermediate polarities ($-1.0 < logP < 1.0$), whereas no highly polar molecules are present.
The study presented in this paper will focus on the compounds that must be extracted and analyzed with a generic confirmatory analysis when a positive result is evidenced during the screening step.

3.2 Sorbent selection

Since the physico-chemical properties of the analytes are quite diverse, a stationary phase able to provide hydrophobic and hydrophilic interactions was initially selected (HLB). Compounds were first extracted using a generic protocol provided by the manufacturer. Each of the three SPE solutions (loading, washing and elutions) was collected, analyzed, and recoveries (expressed in %) were calculated for each compound (Table 2).

Table 2 : Recovery values of all fractions with the HLB sorbent in three loading conditions (pH 2.5, 7 and 12)

compound	HLB pH 2.5			HLB pH 7			HLB pH 12		
	load	wash	elute	load	wash	elute	load	wash	elute
acebutolol	12%	27%	61%	3%	2%	95%	0%	0%	99%
acetazolamide	0%	0%	62%	0%	0%	86%	51%	3%	0%
adrafinil	0%	0%	82%	0%	0%	86%	0%	0%	88%
atenolol	81%	8%	0%	79%	8%	13%	1%	1%	89%
bendroflumethiazide	0%	0%	90%	0%	0%	100%	35%	4%	61%
benzoylecgonine	14%	8%	71%	0%	0%	93%	0%	2%	4%
betamethasone	0%	0%	88%	0%	0%	99%	0%	0%	81%
bumetanide	0%	0%	93%	0%	0%	100%	3%	3%	93%
canrenone	0%	0%	85%	0%	0%	100%	0%	0%	100%
carteolol	45%	24%	26%	1%	0%	94%	0%	0%	90%
celiprolol	1%	6%	89%	0%	0%	97%	0%	0%	97%
chlorothiazide	2%	0%	79%	0%	0%	100%	0%	0%	45%
chlortalidone	0%	0%	100%	0%	0%	100%	66%	6%	20%
clopamide	4%	0%	94%	0%	0%	97%	33%	11%	56%
dexamethasone	0%	0%	100%	0%	0%	99%	0%	0%	62%
dichlorphenamide	0%	0%	86%	0%	0%	100%	93%	0%	0%
esmolol	37%	2%	61%	0%	0%	98%	0%	0%	1%
ethacrynic acid	0%	0%	100%	0%	0%	100%	0%	14%	33%
finasteride	0%	0%	98%	0%	0%	100%	2%	0%	98%
furosemide	0%	0%	100%	0%	0%	93%	0%	0%	100%
gestrinone	0%	0%	95%	0%	0%	97%	0%	0%	83%
hydrochlorothiazide	0%	0%	68%	0%	0%	100%	79%	0%	0%
indapamide	0%	0%	90%	0%	0%	97%	0%	0%	7%
metipranolol	20%	6%	62%	0%	0%	100%	0%	0%	10%
metolazone	0%	0%	93%	0%	0%	83%	0%	0%	77%
metoprolol	9%	20%	63%	6%	6%	88%	0%	0%	89%
modafinil	0%	0%	88%	55%	0%	45%	0%	0%	100%
nadolol	47%	22%	13%	29%	3%	68%	0%	0%	100%
piretanide	0%	0%	96%	0%	0%	98%	20%	14%	49%
probenecid	0%	0%	88%	0%	0%	99%	0%	0%	98%
sotalol	75%	14%	1%	67%	8%	25%	78%	12%	0%
strychnine	0%	0%	82%	0%	0%	99%	0%	0%	83%
torasemide	1%	0%	86%	0%	0%	96%	5%	1%	86%
xipamide	0%	0%	92%	0%	0%	98%	53%	0%	28%

To evaluate the best retention conditions for the loading step, various pH conditions were tested (pH 2.5, 7, and 12). At acidic and neutral pH, basic compounds were inadequately retained. At basic pH, weak acids with low polarity were eluted during the loading step, as evidenced for chlorthalidone (logD = -1.4) and hydrochlorothiazide (logD = -1.8). Oasis HLB was therefore not suitable for quantitative extraction of this compound's set.

In the second step, strong and weak cation exchangers (MCX and WCX, respectively), and strong and weak anion exchangers (MAX and WAX, respectively) were tested. As expected, recoveries from

the various fractions demonstrated that MAX and WAX sorbents were unable to retain basic compounds, whereas the weak cation exchanger (WCX) was unable to keep acidic compounds during the loading step. However, the Oasis MCX sorbent allowed good retention and elution of almost all compounds, and was therefore selected as the sorbent of choice for the remainder of the study (Table 3). This support probably presents the best compromise when a complex mixture of analytes is considered, as demonstrated elsewhere [21]. An optimization of the generic extraction protocol was achieved on the basis of this sorbent selection. In order to rapidly develop a generic method, a restricted number of compounds was selected as representative substances.

Table 3 : Recovery values of all fractions with MCX, WAX, MAX and WCX sorbents.

compound	MCX				WCX			
	load	wash	elute 1	elute 2	load	wash	elute 1	elute 2
acebutolol	0%	0%	0%	98%	0%	0%	16%	59%
acetazolamide	0%	0%	35%	0%	0%	53%	0%	0%
adrafinil	0%	0%	99%	1%	14%	61%	24%	1%
atenolol	0%	0%	31%	69%	0%	0%	64%	13%
bendroflumethiazide	0%	0%	42%	4%	0%	0%	100%	0%
benzoylecgonine	23%	0%	3%	74%	35%	9%	18%	0%
betamethasone	0%	0%	100%	0%	0%	0%	98%	2%
bumetanide	0%	0%	91%	2%	36%	41%	17%	5%
canrenone	0%	0%	100%	0%	0%	0%	100%	0%
carteolol	0%	0%	0%	98%	0%	0%	84%	2%
celiprolol	0%	1%	0%	99%	0%	0%	93%	1%
chlorothiazide	0%	0%	95%	0%	0%	100%	0%	0%
chlortalidone	0%	0%	99%	1%	10%	66%	22%	2%
clopamide	0%	0%	94%	6%	7%	91%	2%	0%
dexamethasone	0%	0%	100%	0%	0%	51%	48%	1%
dichlorphenamide	0%	0%	83%	1%	6%	72%	7%	3%
esmolol	0%	0%	0%	85%	0%	0%	58%	16%
ethacrynic acid	27%	0%	70%	2%	57%	18%	3%	1%
finasteride	0%	0%	97%	3%	0%	0%	97%	3%
furosemide	0%	0%	97%	3%	14%	59%	23%	4%
gestrinone	0%	0%	80%	2%	0%	92%	8%	0%
hydrochlorothiazide	0%	0%	90%	2%	27%	71%	2%	0%
indapamide	0%	0%	98%	2%	0%	24%	45%	1%
metipranolol	0%	0%	0%	100%	0%	0%	42%	58%
metolazone	0%	0%	91%	1%	0%	20%	49%	1%
metoprolol	0%	0%	0%	100%	0%	0%	1%	99%
modafinil	4%	0%	70%	1%	11%	0%	82%	2%
nadolol	0%	0%	0%	100%	0%	98%	0%	2%
piretanide	0%	0%	75%	0%	51%	44%	3%	1%
probenecid	0%	0%	88%	2%	0%	23%	25%	6%
sotalol	0%	0%	0%	100%	0%	95%	1%	4%
strychnine	0%	0%	1%	93%	0%	54%	26%	8%
torasemide	0%	0%	0%	100%	0%	41%	52%	4%
xipamide	0%	0%	97%	3%	10%	68%	14%	8%

compound	WAX				MAX			
	load	wash	elute 1	elute 2	load	wash	elute 1	elute 2
acebutolol	29%	24%	29%	1%	77%	0%	11%	0%
acetazolamide	0%	0%	44%	0%	0%	0%	0%	46%
adrafinil	0%	0%	13%	87%	0%	9%	0%	91%
atenolol	76%	0%	8%	0%	100%	0%	0%	0%
bendroflumethiazide	0%	0%	85%	0%	0%	0%	0%	100%
benzoylecgonine	62%	21%	18%	0%	71%	0%	13%	16%
betamethasone	0%	0%	100%	0%	0%	0%	100%	0%
bumetanide	0%	0%	15%	85%	0%	0%	0%	100%
canrenone	0%	0%	100%	0%	0%	0%	100%	0%
carteolol	44%	28%	16%	0%	94%	0%	5%	1%
celiprolol	36%	24%	33%	0%	78%	0%	14%	0%
chlorothiazide	0%	0%	76%	9%	0%	0%	0%	48%
chlortalidone	0%	0%	98%	2%	0%	0%	5%	87%
clopamide	23%	0%	73%	4%	18%	0%	2%	80%
dexamethasone	0%	0%	97%	0%	0%	0%	61%	18%
dichlorphenamide	0%	0%	79%	3%	0%	0%	0%	71%
esmolol	40%	28%	21%	0%	67%	0%	17%	0%
ethacrynic acid	0%	0%	0%	82%	0%	0%	0%	19%
finasteride	0%	0%	97%	3%	0%	0%	97%	3%
furosemide	0%	0%	13%	87%	0%	3%	0%	97%
gestrinone	0%	0%	75%	0%	0%	0%	80%	2%
hydrochlorothiazide	0%	0%	92%	2%	10%	0%	0%	90%
indapamide	0%	0%	90%	3%	0%	0%	0%	75%
metipranolol	0%	59%	39%	0%	0%	0%	45%	0%
metolazone	0%	0%	81%	1%	0%	0%	0%	71%
metoprolol	49%	21%	28%	2%	84%	0%	15%	1%
modafinil	14%	0%	13%	1%	10%	0%	85%	1%
nadolol	52%	0%	0%	0%	100%	0%	0%	0%
piretanide	0%	0%	9%	81%	0%	0%	0%	84%
probenecid	0%	0%	8%	82%	0%	0%	0%	78%
sotalol	62%	28%	10%	0%	94%	0%	0%	5%
strychnine	35%	27%	18%	0%	59%	0%	5%	0%
torasemide	0%	0%	80%	11%	0%	4%	0%	87%
xipamide	0%	0%	6%	94%	0%	0%	0%	100%

3.3 Selection of representative compounds

For subsequent SPE optimization, representative compounds were selected. To identify groups of compounds presenting similar SPE behavior, it is possible to obtain from the literature physico-chemical data (*i.e.* pKa, logD values) and cluster compounds with the help of chemometric tools as already reported [22]. However, two major restrictions were identified. First, missing values were generally observed and the reliability of data determined by mathematical algorithms could significantly differ from the actual value or be uncertain in cases of particular chemical structures (*e.g.*

cyclization). In this paper, group clustering was achieved owing to the analysis of recoveries for all compounds in each fraction obtained during the sorbent selection step. Due to the large number of values (544), multivariate analysis was achieved on the entire data set with a hierarchical cluster analysis (HCA). Global variation is summarized on a dendrogram, where dissimilarity between scores is indicated *via* the distance between the branches of the tree. The recovery values on all tested sorbents were necessary to obtain better differentiation among compounds than that brought by Oasis MCX only. For the purpose of clarity, compound names as well as therapeutic and chemical families were identified (Figure 1). Four main groups clearly emerged, corresponding to main chemical classes (7 acids, 10 weak acids, 6 neutrals and 11 bases) because these four chemical classes presented different SPE behaviors leading to clear clustering.

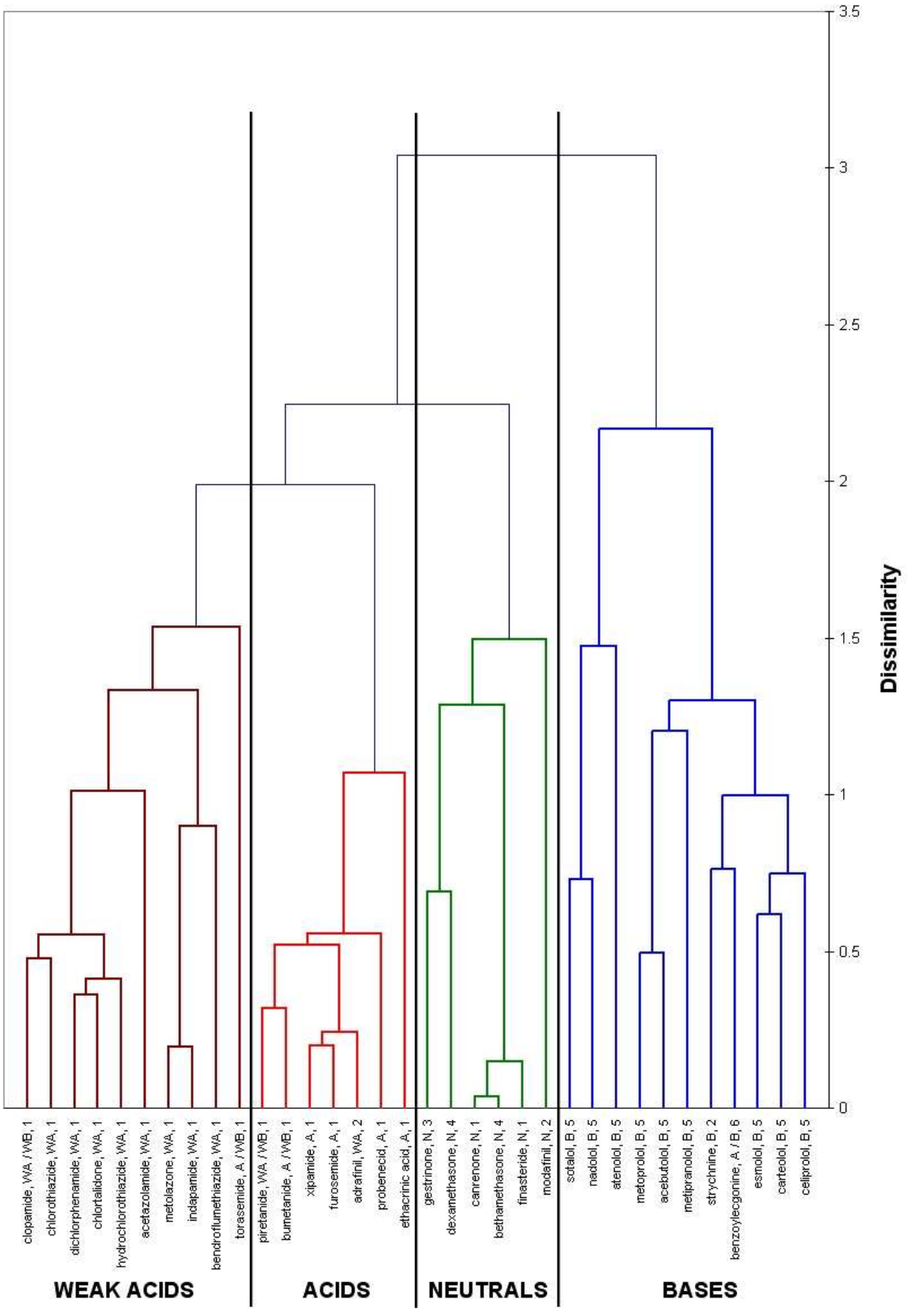

Figure 1 : Dissimilarity dendrogram for SPE behavior. A = acid, B = base, N = neutral, WA = weak acid, WB = weak base, 1 = diuretic, 2 = stimulant, 3 = anabolic agent, 4 = corticosteroid, 5 = β-blocker, 6 = narcotic.

Acids and weak acids were evidenced by a different SPE behavior brought by extraction results from WAX and MAX sorbents. The observed clustering confirmed that evaluation of fundamental physico-chemical properties was the simplest method to determine classification in SPE. Therefore, screening analyses should be preferentially operated by considering the chemical class rather than the therapeutic one. Moreover, the presence of zwitterionic compounds, such as benzoylecgonine, emphasized the importance of clustering compounds by their practical SPE behavior rather than on values from the literature. Indeed, benzoylecgonine behaved like a base, whereas it presents both acidic and basic functions (Figure 2).

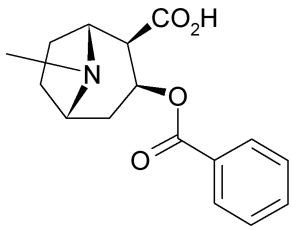

Figure 2 : Benzoylecgonine chemical structure.

Finally, one compound per group was selected for protocol optimization by taking into account logP and logD values. Betamethasone, chlortalidone, ethacrynic acid, and metoprolol were chosen. For instance, the choice of ethacrynic acid was made to characterize the group of acids because it presents average logP (3.38) and logD values (2.9, -0.8, and -1.5, respectively at pH 1, 7, and 10, see Table 1). Betamethasone, chlorthalidone and metoprolol were chosen following the same criteria. Time required for the LC separation of all four representatives was also taken into account. Indeed, optimizing a protocol is time-consuming and must be performed on a large number of compounds. Therefore, representative analytes were chosen to be separated in a short time in the simple isocratic mode.

3.4 Sample preparation optimization

3.4.1 SPE

Each step was optimized with the selected analytes taking into account pH, volume, nature, and proportion of the solvent mixture. Regarding loading, a strong adjustment had to be planned, as urine pH can vary from 4 to 9. Acidification was chosen in agreement with the retention principle of the sorbent and as the most reported pre-treatment for extraction on MCX [23;24]. The sample was therefore loaded after dilution (50:50, v/v) with HCl (240 mM) and several urines with various pH were investigated. At these conditions, no compound loss was observed in the loading step (Table 4).

Table 4 : recovery values in the loading step with four different urines at A) pH 5.3, B) pH 7.8, C) pH 6.8, and D) pH 6.3.

A.

	recovery (%)			mean	RSD
betamethasone	3%	5%	6%	5%	0%
chlortalidone	4%	5%	7%	5%	0%
ethacrynic acid	5%	9%	8%	7%	0%
metoprolol	0%	1%	0%	0%	2%

B.

	recovery (%)			mean	RSD
betamethasone	1%	4%	7%	4%	1%
chlortalidone	6%	2%	3%	4%	1%
ethacrynic acid	7%	9%	6%	7%	0%
metoprolol	0%	0%	0%	0%	0%

C.

	recovery (%)			mean	RSD
betamethasone	2%	7%	4%	4%	1%
chlortalidone	4%	6%	3%	4%	0%
ethacrynic acid	8%	5%	6%	6%	0%
metoprolol	0%	0%	0%	0%	0%

D.

	recovery (%)			mean	RSD
betamethasone	4%	8%	1%	4%	1%
chlortalidone	3%	1%	7%	4%	1%
ethacrynic acid	9%	5%	8%	7%	0%
metoprolol	0%	0%	0%	0%	0%

The washing step was also optimized regarding pH and organic solvent content, and 10% of methanol in washing solution was further used. Finally, both elutions were optimized regarding the volumes used of methanol and basified methanol with 5% ammonia. Increasing volumes of methanol (50 µL, 100 µL, 150 µL, 200 µL, 250 µL, 500 µL, 750 µL, 1000 µL) were tested. It appeared that, for both elutions, a volume of 250 µL of solvent was the lower limit, allowing 100% recovery and this volume was therefore considered optimal.

3.4.2 Reconstitution

Both elutions required a high amount of methanol, which can induce further chromatographic issues. Therefore, the reduction of organic content in injection solvent was tested. As expected, dilution with water while maintaining a constant injection volume was found to be fast and easy, but caused sensitivity loss. Thus, evaporation to dryness was selected for sample concentration and a complete plate required about 2.3 hours (mainly due to the abovementioned evaporation step), corresponding to less than 2 minutes per sample. Reconstitution was evaluated with increasing volumes (25 µL, 50 µL, 100 µL, 150 µL, 200 µL, and 250 µL), and increasing proportions of MeOH (20%, 50% and 100%) and the best compromise was found to be 50 µL of a mixture of water - MeOH (50:50, v/v) (Table 5).

Table 5 : reconstitution yield with 50µL of water/MeOH 50:50.

	recovery (%)			mean	RSD
betamethasone	105%	96%	99%	100%	5%
chlortalidone	102%	95%	98%	98%	4%
ethacrynic acid	98%	100%	99%	99%	1%
metoprolol	96%	104%	98%	99%	4%

Finally, the optimized method required sample centrifugation at 10.000 g for 10 minutes. Seven hundred and fifty microliters of HCl (240 mM) was added to 750 µL of the collected supernatant. The sorbent was conditioned with 500 µL of MeOH and equilibrated with 500 µL of HCl (120 mM). One thousand microliters of the acidified sample was loaded and washed with a mixture of HCl (120 mM) - MeOH (90:10, v/v). First elution was operated with 250 µL of MeOH, and the second elution with the same volume of 5% NH_4OH in MeOH. Both elutions were evaporated to dryness with the same apparatus used in paragraph 2.1 and reconstituted in 50 µL of a mixture of water - MeOH (50:50, v/v). The optimized method was tested on the full set of compounds. Thus, a neat extraction standard of the 34 analytes was extracted in triplicate following the optimized protocol (illustration 1 in Figure 3a). Recoveries were calculated in comparison to a neat standard solution (illustration 2 in Figure 3a) and were comprised in the 80-110% range with repeatability RSD < 10% for all compounds, demonstrating a good method transfer from the four representatives to the full set of analytes.

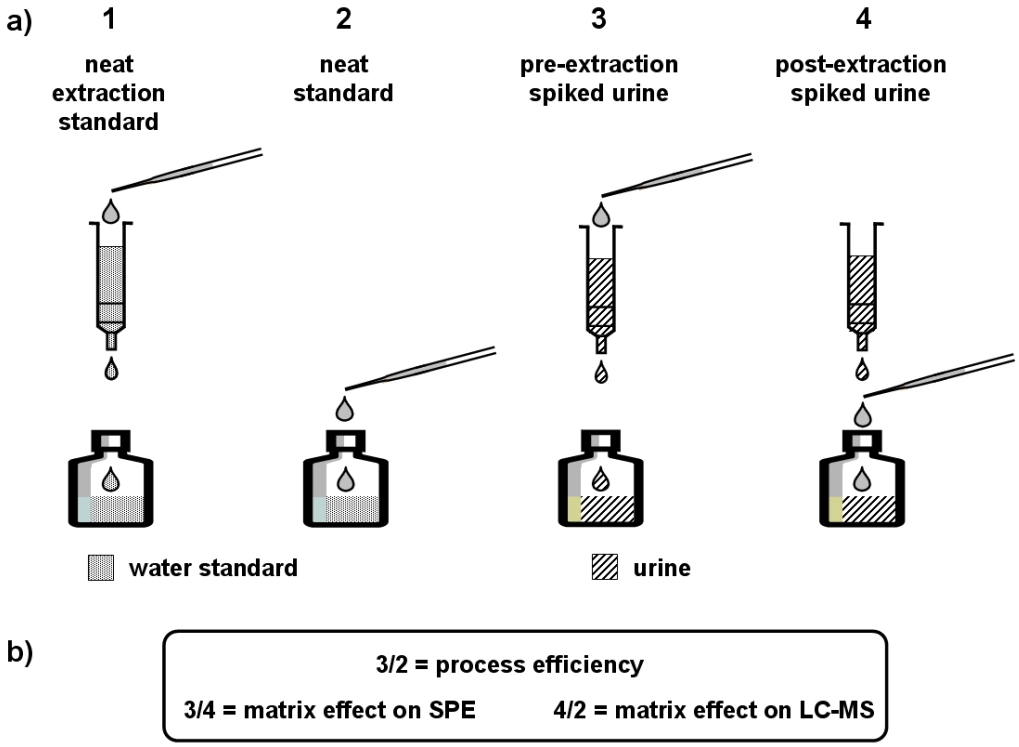

Figure 3 : Schematic illustration of experiments for matrix effects evaluation.

3.5 Matrix effects

Matrix effects were evaluated and their origin determined *via* a method based on Matuszewski *et al.* [18]. First, the process efficiency was considered in urine samples. A urine sample spiked with all analytes was extracted in triplicate (pre-extraction spiked urine, illustration 3 in Figure 3a) and

compared to a neat standard solution (illustration 2 in Figure 3a). Process efficiencies were determined by 3/2 ratios (Figure 3b) and ranged from 10% to 97% with RSD values < 27% (Table 6), indicating an effect of the matrix on the extraction process and/or on MS ionization.

Table 6 : Process efficiency expressed as matrix effect on MS and on SPE with repeatability values (RSD) for each evaluated compound.

compound	process efficiency (RSD)	MS (RSD)	SPE (RSD)
acebutolol	59% (2%)	65% (3%)	90% (4%)
acetazolamide	26% (10%)	28% (6%)	92% (10%)
adrafinil	54% (3%)	55% (2%)	98% (6%)
atenolol	48% (6%)	53% (3%)	91% (7%)
bendroflumethiazide	69% (6%)	72% (2%)	96% (5%)
benzoylecgonine	46% (5%)	49% (8%)	91% (1%)
betamethasone	77% (27%)	72% (33%)	106% (4%)
bumetanide	82% (1%)	86% (5%)	95% (7%)
canrenone	69% (11%)	71% (10%)	99% (5%)
carteolol	64% (4%)	64% (9%)	100% (7%)
celiprolol	71% (3%)	79% (5%)	90% (4%)
chlorothiazide	24% (8%)	26% (5%)	91% (4%)
chlorthalidone	75% (2%)	65% (10%)	114% (10%)
clopamide	35% (2%)	38% (4%)	91% (3%)
dexamethasone	79% (3%)	80% (1%)	98% (3%)
dichlorphenamide	86% (5%)	78% (4%)	109% (6%)
esmolol	10% (12%)	9% (4%)	109% (11%)
ethacrynic acid	52% (8%)	56% (6%)	93% (10%)
finasteride	73% (11%)	75% (12%)	94% (5%)
furosemide	83% (1%)	98% (4%)	85% (3%)
gestrinone	74% (10%)	74% (15%)	100% (5%)
hydrochlorothiazide	62% (2%)	55% (6%)	113% (4%)
indapamide	97% (16%)	94% (13%)	98% (7%)
metipranolol	56% (16%)	61% (5%)	93% (12%)
metolazone	33% (20%)	26% (15%)	111% (10%)
metoprolol	27% (7%)	30% (3%)	89% (7%)
modafinil	96% (11%)	83% (10%)	107% (15%)
nadolol	54% (10%)	53% (2%)	97% (10%)
piretanide	51% (25%)	46% (8%)	93% (9%)
probenecid	93% (3%)	100% (3%)	93% (10%)
sotalol	58% (15%)	51% (6%)	109% (9%)
strychnine	36% (6%)	39% (15%)	94% (9%)
torasemide	57% (8%)	66% (7%)	85% (5%)
xipamide	68% (5%)	67% (2%)	101% (7%)

In order to determine the matrix effect origin for each compound, a blank urine sample was extracted and spiked with the considered analytes (post-extraction spiked urine, illustration 4 in Figure 3a). The

influence of the matrix on the SPE process was determined by 3/4 ratios, since in this case, matrix effects on LC-MS were similar with both samples (all analytes were dissolved in extracted urine). In this study, urine was not considered to affect the extraction process because values of the 3/4 ratio were all comprised between 85% and 114% with repeatability RSD < 15%. Finally, matrix effects on LC-MS were estimated through 4/2 ratios, as they compared the analytical response given by a neat standard and the same solution added in extracted urine. The latter effects were found to contribute the most to the low process efficiency values (Table 6).

However, other situations emerged, such as the case of furosemide, where matrix effects were observed only during the SPE step. Metoprolol and torasemide presented matrix effects during both SPE and analysis steps, while indapamide, modafinil and probenecid were not subjected to any matrix effect. Since various combinations of matrix effects on SPE and on the analysis came out, a classification of all possibilities encountered when a sample preparation is performed prior to the analysis is proposed, still based on the protocol developed by Matuszewski *et al.* The effect of the matrix on SPE can only be negative (-1) or null (0), since analytes can compete for access to interaction sites. Matrix effect on ionization can be negative (-1), null (0), or positive (+), since signal suppression, no signal alteration, or signal enhancement can occur with MS detection. These different situations lead to eight possible cases, summarized in Table 7.

Table 7 : Summary of matrix effect cases (negative effect (-), no effect (0), positive effect (+)).

process efficiency	-1	-1	-1	-1	0	0	1	1
MS	-1	-1	0	1	0	1	1	1
SPE	-1	0	-1	-1	0	-1	-1	0
case	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**

A negative process efficiency can be due to the combination of a negative effect of the matrix on SPE and a signal suppression (case 1), only signal suppression (case 2), only negative effect of the matrix on SPE (case 3) or a combination of a negative effect of the matrix on SPE heavier than signal enhancement (case 4). By the thought process, a good process efficiency around 100% can reflect an absence of matrix effect (case 5), but may also be due to a contribution of a negative effect on the SPE balanced by a signal enhancement (case 6). Finally, a positive process efficiency should be reasonably attributed to signal enhancement exceeding a negative effect on SPE (case 7) or to signal enhancement only (case 8). Thus, process efficiencies, matrix effects on the SPE step, and signal alterations were used to define the situation of each compound, summarized in Table 8.

Table 8 : Matrix effect case reported for each analyte (bold : representative compound).

compound	case	group
acebutolol	1	B
acetazolamide	2	WA
adrafinil	2	A
atenolol	2	B
bendroflumethiazide	2	WA
benzoylecgonine	2	B
betamethasone	2	N
bumetanide	2	A
canrenone	2	N
carteolol	2	B
celiprolol	2	B
chlorothiazide	2	WA
chlortalidone	2	WA
clopamide	2	WA
dexamethasone	2	N
dichlorphenamide	2	WA
esmolol	2	B
ethacrynic acid	2	A
finasteride	2	N
furosemide	3	A
gestrinone	2	N
hydrochlorothiazide	2	WA
indapamide	5	WA
metipranolol	2	B
metolazone	2	WA
metoprolol	1	B
modafinil	5	N
nadolol	2	B
piretanide	2	A
probenecid	5	A
sotalol	2	B
strychnine	2	B
torasemide	1	WA
xipamide	2	A

A reasonable limit was set at ±10% to determine if an effect was significant. It is revealed that there is no correlation between matrix effects and the groups of compounds determined in paragraph 3.2. Therefore, representative compounds cannot be used for matrix effect evaluation, the latter being specific to each compound and needing to be estimated individually. Finally, it must be emphasized that no compound presented signal enhancement, certainly due to the use of the ESI source, which is more susceptible to signal suppression than signal enhancement, explaining the absence of cases 4, 6, 7 and 8.

In conclusion, the extraction of urine samples was found to have only a low influence on the SPE process (4 cases over 34), but endogenous compounds were not completely removed and produced matrix effects during LC-MS analysis. Subsequent quantification would not be affected when deuterated internal standards are used, at least for compounds subject to matrix effects (all compounds except indapamide, modafinil and probenecid). If these standards are not available, quantification should absolutely be achieved within the matrix to prevent from the important matrix effect. Finally, it has to be noted that the optimization of extraction could be rapidly achieved by the help of representative analytes, whereas matrix effects must be evaluated for each analyte, as physico-chemical properties do not allow a good prediction.

4. Conclusion

The main objective of this work was to propose the use of representative compounds to perform easier and faster optimization of sample preparation in the case of multianalytes determination in a complex matrix such as urine. All compounds were first extracted onto different sorbents. From this data set, four main groups of compounds presenting similar SPE behaviors were brought out with the help of a chemometric tool. For each group of analytes, one compound was chosen as representative and used for subsequent protocol optimization. The optimized SPE protocol allows a complete preparation of 96 samples in less than 2 minutes per sample.

The optimized protocol was tested on a neat standard solution of the 34 compounds and provided excellent recovery and repeatability, proving good representation of the entire set by the four selected compounds. Matrix effects were carefully evaluated, and it was determined that most of the compounds were subject to signal suppression, indicating the difficulty in removing interferents when a complex mixture of compounds with various properties has to be extracted. A classification of probable matrix effects encountered during sample preparation prior to the analysis was then proposed, including eight different cases. It emphasized that matrix effects should be evaluated for each compound individually, since representative compounds were not adapted for matrix effect determination.

Acknowledgements

The authors wish to acknowledge Waters for providing the SPE well plates and Anne-Marie Orkild for her advice on sample preparation.

Reference List

[1] Y.Hsieh, M.Chintala, H.Mei, J.Agans, J.M.Brisson, K.Ng, W.A.Korfmacher, Rapid Commun. Mass Spectrom. 15 (2001) 2481.

[2] I.Fu, E.J.Woolf, B.K.Matuszewski, J. Pharm. Biomed. Anal. 18 (1998) 347.

[3] C.Muller, P.Schafer, M.Stortzel, S.Vogt, W.Weinmann, J. Chromatogr. B 773 (2002) 47.

[4] C.G.Enke, Anal. Chem. 69 (1997) 4885.

[5] S.Souverain, C.Eap, J.l.Veuthey, S.Rudaz, Clin. Chem. Lab. Med. 41 (2003) 1615.

[6] D.A.Wells. High Throughput Bioanalytical Sample Preparation: Methods and Automation Strategies. Elsevier, New York, 2003.

[7] P.Lopez-Roldan, M.J.Lopez de Alda, D.Barcelo, Anal Bioanal Chem. 378 (2004) 599.

[8] A.Pena, F.Ruano, M.D.Mingorance, Anal. Bioanal. Chem. 385 (2006) 918.

[9] C.Lopez-Blanco, S.Gomez-Alvarez, M.Rey-Garrote, B.Cancho-Grande, J.Simal-Gandara, Anal. Bioanal. Chem. 384 (2006) 1002.

[10] S.Ito, K.Tsukada, J. Chromatogr. A 943 (2001) 39.

[11] E.T.Gangl, M.Annan, N.Spooner, P.Vouros, Anal. Chem. 73 (2001) 5635.

[12] R.Pascoe, J.P.Foley, A.I.Gusev, Anal. Chem. 73 (2001) 6014.

[13] H.Mei, Y.Hsieh, C.Nardo, X.Xu, S.Wang, K.Ng, W.A.Korfmacher, Rapid Commun. Mass Spectrom. 17 (2003) 97.

[14] J.Zrostlikova, J.Hajslova, J.Poustka, P.Begany, J. Chromatogr. A 973 (2002) 13.

[15] B.K.Choi, A.I.Gusev, D.M.Hercules, Anal. Chem. 71 (1999) 4107.

[16] R.Bonfiglio, R.C.King, T.V.Olah, K.Merkle, Rapid Commun. Mass Spectrom. 13 (1999) 1175.

[17] R.King, R.Bonfiglio, C.Fernandez-Metzler, C.Miller-Stein, T.Olah, J. Am. Soc. Mass Spectrom. 11 (2000) 942.

[18] B.K.Matuszewski, M.L.Constanzer, C.M.Chavez-Eng, Anal. Chem. 75 (2003) 3019.

[19] E.Chambers, D.M.Wagrowski-Diehl, Z.Lu, J.R.Mazzeo, J. Chromatogr. B 852 (2007) 22.

[20] D.W.Brousmiche, J.E.O'Gara, D.P.Walsh, P.J.Lee, P.C.Iraneta, B.C.Trammell, Y.Xu, C.R.Mallet, J. Chromatogr. A 1191 (2008) 108.

[21] B.Kasprzyk-Hordern, R.M.Dinsdale, A.J.Guwy, Anal. Bioanal. Chem. 391 (2008) 1293.

[22] A.Pagliara, E.Khamis, A.Trinh, P.A.Carrupt, R.S.Tsai, B.Testa, J. Liq. Chromatogr. 18 (1995) 1721.

[23] R.Coles, E.D.Kharasch, J. Chromatogr. B 857 (2007) 67.

[24] D.Siluk, D.E.Mager, N.Gronich, D.Abernethy, I.W.Wainer, J. Chromatogr. B 859 (2007) 213.

Article IV

Characterization and classification of matrix effects in biological samples analysis

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en rédaction

ABSTRACT

An exhaustive classification of matrix effects occurring when a sample preparation is performed prior to LC-MS analyses was proposed. A total of eight different situations were identified allowing the recognition of the matrix effect typology *via* the calculation of four recovery values. A set of a hundred and ninety-nine compounds was used to evaluate matrix effect after solid-phase extraction (SPE) from plasma or urine samples prior to LC-ESI-MS analysis. Matrix effect identification was achieved for all compounds and classified through an organization chart. Only 17% of the tested compounds did not present significant matrix effects.

Keywords : matrix effects, human plasma, urine, SPE, MS

1. Introduction

Since the apparition of ionization sources working at atmospheric pressure, liquid-chromatography coupled to mass spectrometry (LC-MS) has become the gold standard for the analysis of pharmaceutical compounds in biological matrices such as blood, plasma, serum and urine [1-6]. As extensively described in the literature, direct injection of such samples cannot be performed due to the presence of endogenous compounds leading to column clogging and MS signal alterations [7-18]. The latter alterations are called matrix effects. Even if not well understood, they have been recognized to happen when interfering substances such as proteins, lipids, sugars or salts co-elute with analytes which could affect the ionization process [11;19;20]. A sample preparation is therefore mandatory to selectively reduce the amount of these interferents. Various methods are available but the most widely used are protein precipitation (PP), liquid-liquid extraction (LLE) and solid phase extraction (SPE). These sample preparations remove the major part of the endogenous material, but a small amount often remains in the treated sample, possibly inducing matrix effects [18;21]. The latter must therefore be tested during method development to determine their presence and impact on the quantification of analytes. Matrix effect evaluation can be achieved *via* two techniques providing complementary information. First, qualitative results can be obtained with a post-column infusion system as proposed in 1999 by Bonfiglio *et al.* [22]. The principle is based on the infusion of a solution of analytes of interest between the column and the MS detector, leading to a constant baseline. Blank samples, extracted with the tested sample preparation procedure, are injected in the system. The presence of matrix effects is highlighted by baseline alteration in a time window. MS responses of analytes eluting within this region will be altered (signal suppression or enhancement), inducing irreproducible and non quantitative results. The second strategy for matrix effect evaluation was proposed by Matuszewski *et al.* in 2003 [23] and leads to a quantitative information. This method determines if the presence of matrix interferents causes a problem during the sample preparation step and/or during the analysis, based on the comparison of three different samples: a neat standard, a biological sample fortified prior the extraction and a biological sample fortified after the extraction.
Since matrix effects can occur during the sample preparation and/or the analysis, various situations can arise. To the best of our knowledge, no exhaustive investigation of the various matrix effects was published. The aim of this study was therefore to build an exhaustive classification of probable matrix effects encountered when a sample preparation is performed prior to a LC-MS analysis and to test the validity of the proposed model on a set of 199 compounds of pharmaceutical or doping interest.

2. Experimental

The influence of the matrix on the SPE procedure and the LC-MS analysis was evaluated on a hundred and ninety-nine compounds in urine and plasma samples. Three different SPE and LC-MS procedures were used.

2.1 Chemicals

All standards came from various pharmaceutical companies and were of pharmaceutical purity. A stock solution at 1 $mg \cdot mL^{-1}$ was prepared for each substance in a mixture of water / ACN (1/1, v/v) and stored at -20℃. Working solutions were made fr om the stock solutions, each one containing from two to six analytes at a concentration below the toxic level. Dilution of stock solution was operated with ultra-pure water generated with a Milli-Q plus water purification system from Millipore (Bedford, USA). Methanol Absolute HPLC-Supra gradient and acetonitrile HPLC-S gradient grade (Biosolve, Valkenswaard, Netherlands), ammonia solution 25% puriss p.a., formic acid puriss p.a. and sodium formate puriss p.a. for HPLC (Fluka, Buchs, Switzerland), and hydrochloric acid fuming 37% puriss p.a. (Merck, Darmstadt, Germany) were used in all experiments. Human plasma and urine samples were obtained from a total of six healthy non-drug-consuming volunteers.

2.2 SPE

2.2.1 Oasis HLB

Oasis HLB 30 mg cartridges with 30 µm particles (Waters Corporation, Milford, MA, USA) were employed to prepare plasma samples. An automated ASPEC GX-274 (Gilson, Middletown, USA) system was employed to manage SPE cartridges. One millilitre of MeOH and 1 ml of HCl 6N 2% in water was used for sorbent conditioning and equilibration. One millilitre of sample was loaded and washing was performed with 1 ml of a mixture of HCl 120 mM / MeOH (90/10, v/v). Elution was finally carried out with 500 µL of MeOH. Elution solutions were directly transferred to injection vials.

2.2.2 Oasis MAX

Oasis MAX 30 mg cartridges with 30 µm particles (Waters Corporation) were employed to extract acidic compounds from urine samples. They were centrifuged at 2500 g for 5 min and 750µL of 5% NH_4OH was added to 750 µL of the supernatant. The sorbent was conditioned with 500 µL of MeOH and equilibrated 500 µL of 5% NH_4OH. One millilitre of the basified sample was loaded and washing was carried out with 1 mL of 5% NH_4OH and then with 250 µL of MeOH. Elution was performed with 250 µL of 2% HCOOH in MeOH. Elution solutions were directly transferred to injection vials.

2.2.3 Oasis MCX

Oasis MCX 30 mg cartridges with 30 µm particles (Waters Corporation) were used to extract basic and neutral compounds from urine samples. They were centrifuged at 2500 g for 5 minutes and 750 µL of HCl 240 mM was added to 750 µL of the collected supernatant. The sorbent was conditioned with 500 µL of MeOH and equilibrated with 500 µL of HCl 120 mM. One millilitre of the acidified sample was loaded and washed with a mixture of HCl 120 mM / MeOH (90/10, v/v). First elution was operated with 250 µL of MeOH and the second elution with the same volume of 5% NH_4OH in MeOH. Both elutions were evaporated to dryness and reconstituted in 50 µL of a mixture of water / MeOH (50/50, v/v) before the analysis.

2.3 LC-MS

2.3.1 LC-MS

Urine samples extracted on Oasis MCX were analyzed with an Agilent Series 1100 LC system (Agilent Technologies) equipped with an autosampler and a binary pump. This system was coupled to a 1100 MSD single quadrupole (Agilent Technologies) with an orthogonal ESI source.
Five microliters of the sample was injected on a XBridge Shield 100 x 2.1 mm, 3.5 µm column (Waters Corporation) kept at 30℃. The mobile phase was constituted of acetate buffer 20 mM pH 5 / ACN (67/33, v/v) and was delivered in the isocratic mode at 300 $\mu L \cdot min^{-1}$. Nitrogen was used as both nebulizing (5 $L \cdot min^{-1}$) and drying gas (250℃). Vaporizer temperature was set at 250℃, nebulizer pressure at 45 psig and capillary voltage at +2 kV. Detection of protonated analytes was always conducted in the selected ion monitoring (SIM) mode. The Chemstation A.10.03 software (Agilent Technologies) was used for instrument control, data acquisition and data handling.

2.3.2 LC-MS/MS

Plasma samples extracted on Oasis HLB were analyzed with an Agilent Series 1200 LC system (Agilent Technologies, Waldbronn, Germany) equipped with an autosampler, a binary pump and a column heater. This system was coupled to a QTrap system (Applied Biosystems, Darmstadt, Germany) with a TurboIonSpray source.
Ten microliters of the sample was injected on a Hypersil Gold 100mm x 2.1 mm, 1.9 µm column (Thermo-Scientific, Waltham, MA, USA) kept at at 40℃ and equipped with a 2 µm precolumn filter. The mobile phase was delivered at 400 $\mu L \cdot min^{-1}$ in the gradient mode. It was constituted of (A) 0.1% formic acid and sodium formate 1 µM / (B) ACN. An initial mobile phase composition of 5% A and gradually increased to 70% in 12 min, then to 100% in 2 min and finally held at 100% for 1 min. A 5 minutes post-run equilibration time was applied after each analysis. Total eluent flow from the HPLC was directed into the turbo ionspray source without any splitting device. MS detection was performed

in the positive mode and the needle voltage was +5 kV. The collision cell gas (nitrogen) was set at 5 mTorr. The turbo ion spray heater was maintained at 420°C with the nebulizer gas and heater gas set at 35 and 65 psi, respectively. Data acquisition, data handling and instrument control were performed with Analyst® version 1.4.

2.3.3 UPLC-MS

Urine samples extracted on Oasis MCX and MAX were analyzed with a Waters Aquity UPLC system (Waters Corporation) equipped with a binary solvent manager, a cooled auto-sampler kept at 4°C. This system was coupled to a Micromass-Q-Tof Premier® mass spectrometer (Waters Corporation) with an electrospray ionization (ESI) source.

Ten microliters of the sample was injected on a Waters Acquity UPLC BEH C18 2.1 x 50 mm, 1.7 µm column (Waters Corporation) kept at 30°C. The mobile phase was constituted of 0.1% formic acid in water / 0.1% formic acid in ACN, linearly programmed from 5 to 95% B in 3 min, with 1.5 min equilibration time and delivered at 400 $\mu L \cdot min^{-1}$. A Waters Acquity Van Guard BEH C18 2.1 x 5 mm, 1.7 µm precolumn (Waters Corporation) was placed in front of the analytical column. Samples extracted on the MCX sorbent were monitored in the positive mode whereas those extracted on the MAX were monitored in the negative mode. The desolvation gas was delivered at 800 $L \cdot h^{-1}$ and 300°C, capillary was set at +3 kV and -2.4 kV in the positive and negative mode, respectively. The micro-channel plates (MCP) were operated at +1.8 kV -1.7 kV in the positive and negative mode, respectively. The source was adjusted at 100°C, the cone voltage at 40 V and the cone gas flow at 10 $L \cdot h^{-1}$, the collision energy at 5 eV, and the collision gas flow at 0.32 $mL \cdot min^{-1}$ in positive mode and 0.25 $mL \cdot min^{-1}$ in the negative mode. Data acquisition, data handling and instrument control were performed with MassLynx® Software (Waters Corporation).

3. Results and discussion

As stated in 2001 FDA Guidelines, "*In the case of LC-MS/MS-based procedures, appropriate steps should be taken to ensure the lack of matrix effects throughout the application of the method*" [24]. For this purpose, several approaches for matrix effects evaluation were described and, among them, Matuszewski *et al.* [23] proposed a procedure to quantitatively determine on which part of the analytical process matrix effects take place. The quality of the whole analytical process (i.e. *process efficiency*, PE) is related to alterations due to interferences during the sample preparation and analysis (e.g. ionization, ion transmission, etc.). PE was estimated by Matuszewski *et al.* with the ratio of peak areas from a matrix sample fortified prior the sample preparation (***c***, Figure 1) and a neat standard (***a***, Figure 1).

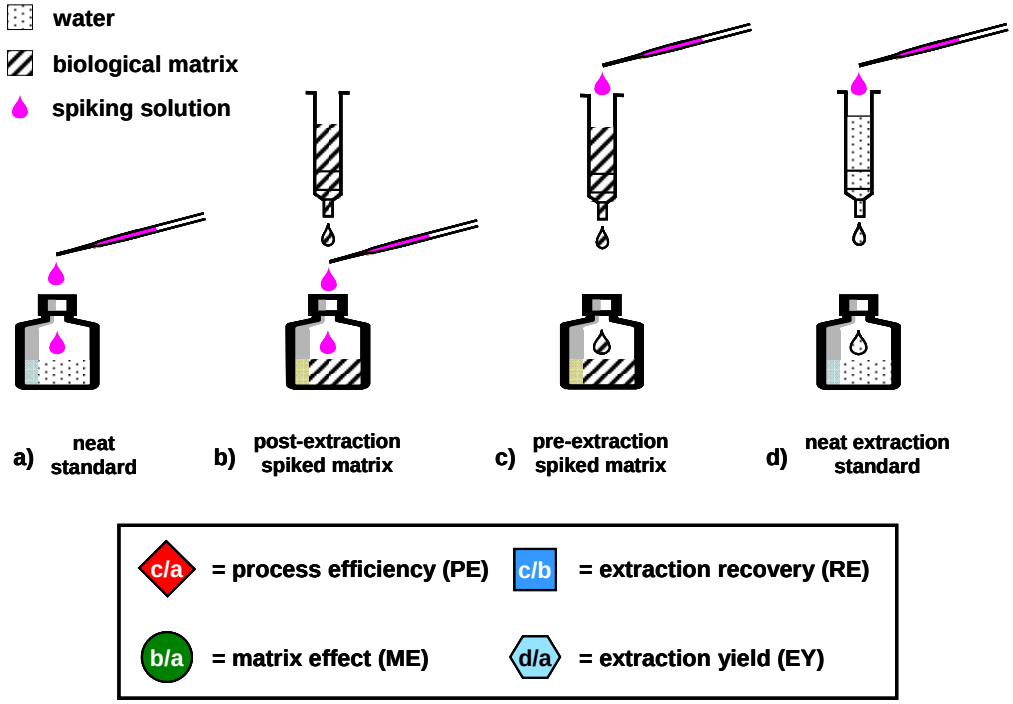

Figure 1 : representation of samples required for matrix effects evaluation and calculation of the required ratios.

Residual compounds still present after sample preparation can interfere with the MS ionization process, leading to the well-known signal suppression or enhancement situations. The influence of endogenous compounds on the LC-MS (i.e. *matrix effect*, ME) is evaluated by the ratio of peak areas from a matrix sample fortified after the sample preparation (***b***, Figure 1) and a neat standard ***a***. This is related to the concept of Matrix Factor (MF), defined as "*a ratio of the analyte peak response in the presence of matrix ions to the analyte peak response in the absence of matrix ions*" in the 2007 Washington workshop/conference report [25].

As previously mentioned, interferents may also affect the sample preparation, leading mostly to a decrease of the extraction yield when compared to standards. The quality of extraction in presence of the matrix (i.e. *extraction recovery*, RE) should therefore be determined with the ratio of peak areas from ***c*** and ***b***. However, this calculation does not allow differentiating a low extraction recovery due to interfering compounds from a poor extraction yield due to the sample preparation itself (i.e. *extraction yield*, EY). Since generic procedures are often used in multianalyte determination, a low extraction yield could lead to a RE misevaluation. This modality was therefore included in this study and required only one additional sample, namely a neat extraction standard (***d***, Figure 1). EY was evaluated with the ratio of peak areas from ***d*** and ***a***. Thus, a complete investigation of matrix effects required only four samples and the calculation of four ratios. Since RE and ME might present various combinations for a given PE, an exhaustive listing of possibilities is proposed in this work.

3.1 Description

As presented in Table 1, PE might be low, good or high (symbolized by -1, 0 and +1, respectively), as a combination of ME and RE. ME can also be divided into three modalities since signal diminution, no alteration or signal enhancement (-1, 0 and +1, respectively), could be observed when endogenous compounds coelute with the analytes. RE presents only two modalities since the sample preparation can reasonably reduce or not influence the extraction recovery (-1 and 0, respectively).

Table 1 : summary of cases (negative effect (-1), no effect (0), positive effect (+1)).

PE	**-1**				**0**		**+1**	
ME	**-1**	**-1**	**0**	**+1**	**0**	**+1**	**+1**	**+1**
RE	**-1**	**0**	**-1**	**-1**	**0**	**-1**	**-1**	**0**
	PE^{-}_{-}	PE^{-}_{0}	PE^{0}_{-}	PE^{+}_{-}	PE^{o}_{o}	PE^{+}_{-}	PE^{+}_{-}	PE^{+}_{0}

According to this, eight combinations could be obtained. For sake of clarity and to define the influence of the matrix on PE, the exponent expresses ME while the suffix expressed RE. Therefore, a PE^{-}_{-} indicates that both matrix effect (signal suppression) and a low extraction recovery were observed.
As indicated in Table 1, a low PE can result from three situations:

1. Pure effect; a low ME (e.g. MS signal suppression) or a low RE (PE^{-}_{0} and PE^{0}_{-}, respectively).

2. Synergistic effect; a simultaneous low ME and RE (PE^{-}_{-}) and 3. Antagonistic effect; a low RE partially compensated by a high ME (e.g. MS signal enhancement) (PE^{+}_{-}).

A good PE results from two different cases:

1. Absence of effect; no matrix influence on both ME and RE (PE^{0}_{0}) and 2. Antagonistic effect; a low RE compensated by a high ME (PE^{+}_{-}).

Finally, a high PE always requires a high ME which can be partially compensated or not by a low RE (PE^{+}_{-} and PE^{+}_{0}, respectively).

These eight combinations could be determined thanks to the procedure proposed by Matuszewski *et al.* However, the five cases presenting a low RE require the evaluation of EY to evidence a possible low extraction yield as indicated in Figure 2. This general scheme was employed to guide the estimation of the matrix influence and the attribution of cases to tested compounds. PE value is first used. The left branch of the scheme corresponds to low PE indicating a significant influence of endogenous compounds on the analytical process. The middle branch represents combinations leading to a good PE but maybe hiding two compensating effects (PE^{+}_{-}). Finally, the right branch reveals, as well as for low PE, a significant influence of endogenous compounds on the analysis. Then, the second row of the scheme allows refining the matrix effect typology, based on the same

principle than discussed for PE. RE is finally used to define precisely the matrix effect typology. Finally, in case of a low RE, EY should be determined.

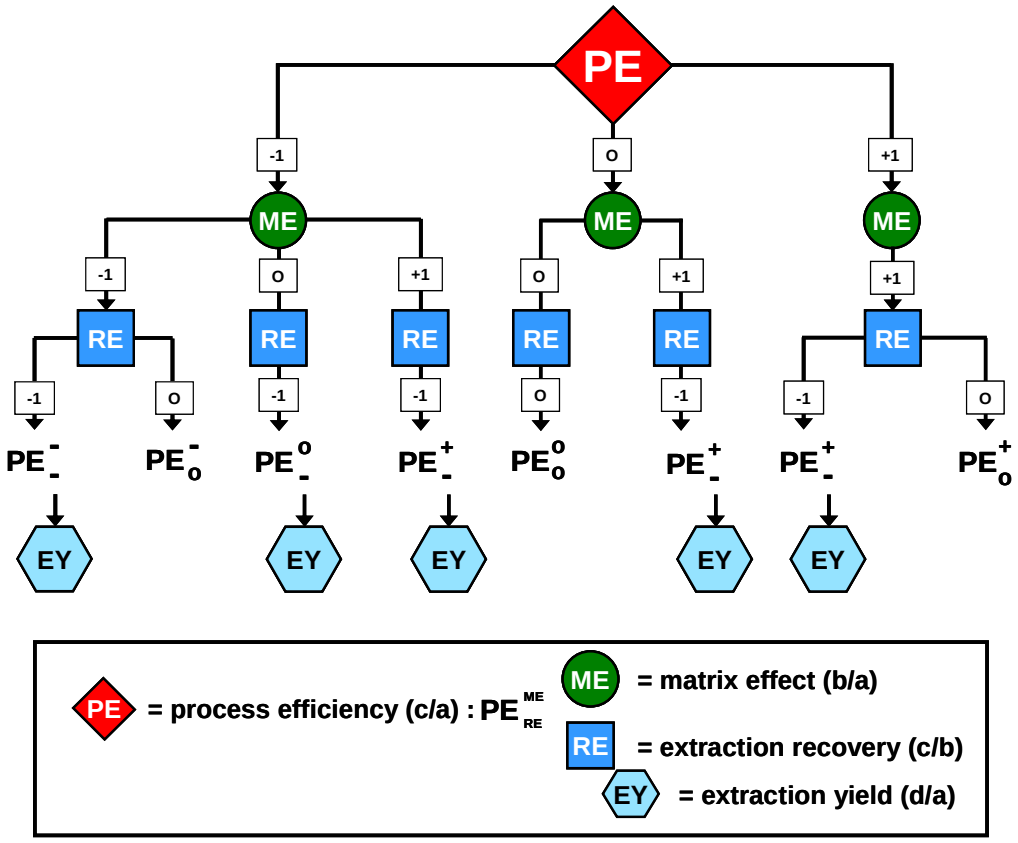

Figure 2 : general scheme for matrix effects attribution.

3.2 Application

This approach was evaluated in regard to its ability to attribute the matrix effect typology to a set of a hundred and ninety-nine analytes (N=199). Data was acquired and all ratios (PE, ME, RE and EY) were determined using two matrices (plasma and urine) with various SPE protocols and LC-MS apparatus. PE and, ME and RE were evaluated in triplicate for all compounds and completed with the estimation of EY (Table 2). Since LC-MS variability was around 5%, a reasonable limit was set at a value of ± 10% to distinguish between low (+1) and high (-1) effects from good situations (0).

Table 2 : list of the hundred and ninety-nine compounds with their respective PE, ME, RE and EY values.

compound	matrix	apparatus	PE	ME	RE	EY
Alprazolam	plasma	LC-MS/MS	45%	49%	90%	82%
Amfepramone	plasma	LC-MS/MS	93%	92%	103%	101%
Amisulpride	plasma	LC-MS/MS	93%	132%	74%	91%
Amitriptyline	plasma	LC-MS/MS	54%	61%	91%	100%
Amphetamine	plasma	LC-MS/MS	78%	91%	86%	89%
Aripirazole	plasma	LC-MS/MS	80%	102%	78%	99%
Atenolol	plasma	LC-MS/MS	44%	92%	48%	34%
Bisoprolol	plasma	LC-MS/MS	101%	99%	102%	96%
Bromazepam	plasma	LC-MS/MS	79%	89%	91%	91%
Buprenorphine	plasma	LC-MS/MS	89%	109%	84%	96%
Bupropion	plasma	LC-MS/MS	109%	134%	83%	91%
Chlordiazepoxide	plasma	LC-MS/MS	93%	91%	103%	95%
Chloroquine	plasma	LC-MS/MS	107%	129%	84%	88%
Chlorprothixène	plasma	LC-MS/MS	36%	53%	71%	90%
Citalopram	plasma	LC-MS/MS	90%	109%	82%	94%
Clobazam	plasma	LC-MS/MS	38%	40%	94%	66%
Clomipramine	plasma	LC-MS/MS	62%	95%	65%	101%
Clonazepam	plasma	LC-MS/MS	34%	41%	83%	86%
Clonidine	plasma	LC-MS/MS	95%	128%	76%	100%
Clotiapine	plasma	LC-MS/MS	29%	33%	90%	105%
Clozapine	plasma	LC-MS/MS	92%	104%	89%	82%
Cocaïne	plasma	LC-MS/MS	92%	97%	96%	93%
Codéine	plasma	LC-MS/MS	85%	105%	81%	16%
Cotinine	plasma	LC-MS/MS	2%	32%	6%	8%
Cyclizine	plasma	LC-MS/MS	95%	109%	88%	94%
Desipramine	plasma	LC-MS/MS	47%	45%	105%	119%
Desmethyl-Chlordiazepoxide	plasma	LC-MS/MS	3%	7%	47%	7%
Dextrometrophane	plasma	LC-MS/MS	77%	89%	86%	99%
Dimetindène	plasma	LC-MS/MS	46%	65%	71%	109%
Diphenhydramine	plasma	LC-MS/MS	84%	111%	77%	56%
Duloxetine	plasma	LC-MS/MS	29%	42%	75%	71%
EDDP	plasma	LC-MS/MS	76%	88%	85%	83%
Flumazenil	plasma	LC-MS/MS	88%	110%	81%	93%
Flunitrazepam	plasma	LC-MS/MS	28%	43%	66%	72%
Fluoxetine	plasma	LC-MS/MS	53%	63%	85%	58%
Fluphenazine	plasma	LC-MS/MS	73%	84%	87%	101%
Fluvoxamine	plasma	LC-MS/MS	74%	103%	72%	98%
Haloperidol	plasma	LC-MS/MS	89%	96%	93%	96%
Héroïne	plasma	LC-MS/MS	107%	248%	44%	93%
9-Hydroxy-Risperidone	plasma	LC-MS/MS	92%	107%	86%	83%
Imipramime	plasma	LC-MS/MS	36%	73%	50%	104%
Indométhacine	plasma	LC-MS/MS	24%	55%	44%	57%
Lamotrigine	plasma	LC-MS/MS	89%	116%	77%	102%
Levopromazine	plasma	LC-MS/MS	14%	26%	56%	59%
Lidocaïne	plasma	LC-MS/MS	93%	114%	83%	84%
Loperamide	plasma	LC-MS/MS	81%	109%	76%	102%
LSD	plasma	LC-MS/MS	55%	67%	82%	90%
Maprotiline	plasma	LC-MS/MS	56%	68%	82%	80%
MDMA (Extasy)	plasma	LC-MS/MS	89%	121%	74%	95%
Methadone	plasma	LC-MS/MS	43%	83%	52%	87%
Methaqualone	plasma	LC-MS/MS	37%	48%	80%	94%
Metoclopramide	plasma	LC-MS/MS	99%	104%	96%	90%
Metoprolol	plasma	LC-MS/MS	84%	106%	79%	94%
Mianserine	plasma	LC-MS/MS	111%	128%	89%	98%
Mirtazapine	plasma	LC-MS/MS	80%	102%	79%	99%

Moclobémide	plasma	LC-MS/MS	85%	96%	89%	90%
6-mono-acetyl-morphine	plasma	LC-MS/MS	103%	102%	103%	89%
Morphine	plasma	LC-MS/MS	15%	33%	45%	23%
N-Desmethyl-Citalopram	plasma	LC-MS/MS	86%	114%	78%	97%
N-Desmethyl-Clomipramine	plasma	LC-MS/MS	68%	89%	76%	98%
N-Desmethyl-Clozapine	plasma	LC-MS/MS	100%	121%	82%	93%
N-Desmethyl-Mirtazapine	plasma	LC-MS/MS	67%	96%	71%	78%
Nefazodone	plasma	LC-MS/MS	70%	92%	76%	86%
Nicotine	plasma	LC-MS/MS	8%	25%	33%	2%
Nitrazepam	plasma	LC-MS/MS	62%	73%	86%	89%
Nordiazepam	plasma	LC-MS/MS	55%	63%	87%	99%
Norfluoxétine	plasma	LC-MS/MS	34%	57%	59%	91%
Nortriptyline	plasma	LC-MS/MS	29%	33%	89%	90%
Noscapine	plasma	LC-MS/MS	96%	115%	84%	90%
O-desmethyl-Venlafaxine	plasma	LC-MS/MS	95%	96%	101%	99%
Olanzapine	plasma	LC-MS/MS	106%	110%	100%	88%
Opipramol	plasma	LC-MS/MS	113%	138%	82%	83%
Orphenadrine	plasma	LC-MS/MS	71%	86%	82%	104%
Oxazepam	plasma	LC-MS/MS	58%	82%	72%	81%
Papaverine	plasma	LC-MS/MS	101%	123%	83%	94%
Paroxetine	plasma	LC-MS/MS	79%	102%	79%	90%
Pentazocine	plasma	LC-MS/MS	95%	98%	98%	85%
Propanolol	plasma	LC-MS/MS	83%	92%	91%	86%
Propofol	plasma	LC-MS/MS	44%	62%	71%	82%
Protriptyline	plasma	LC-MS/MS	49%	74%	67%	103%
Quinine	plasma	LC-MS/MS	32%	37%	88%	118%
Quetiapine	plasma	LC-MS/MS	93%	107%	88%	105%
Reboxetine	plasma	LC-MS/MS	97%	108%	90%	87%
Risperidone	plasma	LC-MS/MS	88%	96%	92%	90%
Sertindole	plasma	LC-MS/MS	99%	122%	82%	103%
Sertraline	plasma	LC-MS/MS	49%	73%	68%	87%
Sotalol	plasma	LC-MS/MS	17%	98%	18%	60%
Tamoxifène	plasma	LC-MS/MS	58%	102%	57%	82%
Thioridazine	plasma	LC-MS/MS	95%	91%	116%	86%
Tramadol	plasma	LC-MS/MS	89%	98%	93%	96%
Triazolam	plasma	LC-MS/MS	78%	79%	99%	99%
Trimipramine	plasma	LC-MS/MS	32%	48%	68%	86%
Venlafaxine	plasma	LC-MS/MS	98%	119%	83%	105%
Verapamil	plasma	LC-MS/MS	63%	75%	85%	97%
Zolpidem	plasma	LC-MS/MS	106%	109%	97%	101%
Zopiclone	plasma	LC-MS/MS	80%	102%	81%	91%
Acetazolamide	urine	UPLC-MS	65%	57%	115%	108%
Amfepramone	urine	UPLC-MS	87%	95%	92%	90%
Amfetaminil	urine	UPLC-MS	47%	83%	57%	33%
Amiloride	urine	UPLC-MS	62%	59%	106%	83%
Anastrozole	urine	UPLC-MS	32%	102%	31%	1%
Anastrozole	urine	UPLC-MS	82%	102%	80%	98%
Atenolol	urine	UPLC-MS	62%	96%	65%	87%
Bendrofluméthiazide	urine	UPLC-MS	99%	153%	64%	90%
Benzoylecgonine	urine	UPLC-MS	111%	100%	111%	87%
Bromantan	urine	UPLC-MS	100%	349%	29%	95%
Buprenorphine	urine	UPLC-MS	105%	145%	72%	47%
Bupropion	urine	UPLC-MS	120%	108%	111%	114%
Caffeine	urine	UPLC-MS	77%	124%	63%	100%
Cathine	urine	UPLC-MS	89%	179%	50%	80%
Celiprolol	urine	UPLC-MS	119%	99%	120%	101%
Chlorothiazide	urine	UPLC-MS	46%	71%	64%	59%
Chlorphentermine	urine	UPLC-MS	91%	106%	85%	119%
Chlorthalidone	urine	UPLC-MS	43%	41%	105%	92%
Clopamide	urine	UPLC-MS	81%	74%	110%	74%
Crothetamide	urine	UPLC-MS	47%	218%	22%	115%

Dextromoramide	urine	UPLC-MS	97%	121%	80%	85%
Dichlorphenamide	urine	UPLC-MS	57%	48%	118%	87%
Dimetamphetamine	urine	UPLC-MS	94%	108%	87%	101%
Ephedrine	urine	UPLC-MS	78%	72%	108%	73%
Etafedrine	urine	UPLC-MS	63%	125%	50%	76%
Ethacrynic acid	urine	UPLC-MS	68%	65%	105%	69%
Ethylamphetamine	urine	UPLC-MS	81%	156%	52%	71%
Etilefrine	urine	UPLC-MS	88%	77%	115%	108%
Fenfluramine	urine	UPLC-MS	113%	160%	71%	113%
Fenproporex	urine	UPLC-MS	65%	121%	54%	83%
Fentanyl	urine	UPLC-MS	105%	105%	100%	109%
Hydrochlorothiazide	urine	UPLC-MS	20%	31%	63%	66%
Indapamide	urine	UPLC-MS	77%	78%	99%	108%
Isometheptene	urine	UPLC-MS	72%	157%	46%	83%
MDA	urine	UPLC-MS	56%	105%	54%	72%
MDMA	urine	UPLC-MS	97%	153%	63%	107%
Mefenorex	urine	UPLC-MS	104%	183%	57%	104%
Métamphetamine	urine	UPLC-MS	71%	93%	76%	67%
Méthadone	urine	UPLC-MS	61%	57%	108%	84%
Methylamphetamine	urine	UPLC-MS	81%	89%	91%	78%
Methylecgonine	urine	UPLC-MS	6%	83%	8%	15%
Méthylphénidate	urine	UPLC-MS	94%	166%	57%	101%
Metipranolol	urine	UPLC-MS	100%	114%	87%	114%
Metolazone	urine	UPLC-MS	46%	229%	20%	87%
Metoprolol	urine	UPLC-MS	101%	86%	117%	97%
Nadolol	urine	UPLC-MS	100%	92%	109%	106%
Nikéthamide	urine	UPLC-MS	62%	75%	83%	76%
Norbuprénorphine	urine	UPLC-MS	37%	70%	53%	46%
Norfentanyl	urine	UPLC-MS	105%	219%	48%	119%
Oxilofrine	urine	UPLC-MS	81%	97%	84%	102%
Pemoline	urine	UPLC-MS	81%	100%	81%	64%
Pentetrazol	urine	UPLC-MS	31%	47%	66%	75%
Phendimetrazine	urine	UPLC-MS	81%	95%	85%	90%
Phenpromethamine	urine	UPLC-MS	93%	204%	46%	110%
Phentermine	urine	UPLC-MS	88%	213%	41%	96%
Phénylpropanolamine	urine	UPLC-MS	85%	105%	81%	110%
Pholedrine	urine	UPLC-MS	78%	86%	91%	95%
Probenecide	urine	UPLC-MS	77%	450%	17%	93%
Prolintane	urine	UPLC-MS	105%	183%	57%	85%
Propylhexedrine	urine	UPLC-MS	77%	152%	51%	79%
Pseudoephedrine	urine	UPLC-MS	80%	75%	107%	89%
Ritalinic acid	urine	UPLC-MS	48%	100%	49%	90%
RSR 13	urine	UPLC-MS	85%	124%	69%	73%
Salmeterol	urine	UPLC-MS	94%	99%	95%	78%
Sibutramine	urine	UPLC-MS	109%	382%	28%	87%
Strychnine	urine	UPLC-MS	55%	79%	70%	81%
Xipamide	urine	UPLC-MS	94%	115%	82%	91%
Acebutolol	urine	LC-MS	59%	65%	90%	93%
Acetazolamide	urine	LC-MS	26%	28%	92%	31%
Adrafinil	urine	LC-MS	54%	55%	98%	98%
Atenolol	urine	LC-MS	48%	53%	91%	86%
Bendroflumethiazide	urine	LC-MS	69%	72%	96%	106%
Benzoylecgonine	urine	LC-MS	46%	49%	91%	92%
Bethamethasone	urine	LC-MS	77%	72%	106%	105%
Bumetanide	urine	LC-MS	82%	86%	95%	84%
Canrenone	urine	LC-MS	69%	71%	99%	89%
Carteolol	urine	LC-MS	64%	64%	100%	93%
Celiprolol	urine	LC-MS	71%	79%	90%	98%
Chlorothiazide	urine	LC-MS	24%	26%	91%	91%
Chlortalidone	urine	LC-MS	75%	65%	114%	86%
Clopamide	urine	LC-MS	35%	38%	91%	93%

Dexamethasone	urine	LC-MS	79%	80%	98%	109%
Dichlorphenamide	urine	LC-MS	86%	78%	109%	107%
Esmolol	urine	LC-MS	10%	9%	109%	95%
Ethacrinic acid	urine	LC-MS	52%	56%	93%	96%
Fentanyl	urine	LC-MS	91%	97%	94%	91%
Finasteride	urine	LC-MS	73%	75%	94%	95%
Furosemide	urine	LC-MS	83%	98%	85%	84%
Gestrinone	urine	LC-MS	74%	74%	100%	99%
Hydrochlorothiazide	urine	LC-MS	62%	55%	113%	106%
Indapamide	urine	LC-MS	97%	94%	98%	103%
Methylphenidate	urine	LC-MS	78%	95%	83%	92%
Metipranolol	urine	LC-MS	56%	61%	93%	99%
Metolazone	urine	LC-MS	33%	26%	111%	98%
Metoprolol	urine	LC-MS	27%	30%	89%	94%
Modafinil	urine	LC-MS	96%	83%	107%	100%
Nadolol	urine	LC-MS	54%	53%	97%	94%
Piretanide	urine	LC-MS	51%	46%	93%	108%
Probenecide	urine	LC-MS	93%	100%	93%	102%
Sotalol	urine	LC-MS	58%	51%	109%	97%
Strychnine	urine	LC-MS	36%	39%	94%	96%
Torasemide	urine	LC-MS	57%	66%	85%	87%
Xipamide	urine	LC-MS	68%	67%	101%	109%

Influence of biological matrix was successfully attributed to each tested analyte according to Figure 2. However, in few situations, PE, ME, RE or EY values were close but not into the acceptance limit, involving unclassified cases. In such situations, the closer matrix effect case was therefore attributed. For example, cyclizine presented good PE, ME and EY values, while RE was 2% lower than the limit (i.e. 88%). This molecule was thus considered to present a good SPE recovery and was then attributed to $PE\,{}^{0}_{0}$ case.

In order to give a clear overview of the results, the number of analytes per case and their related percentage were reported in Figure 3. About two third of the analytes (138 cases, 69%) presented a low PE among which 17 compounds (9%) even revealed a PE lower than 30%, mainly due to the combination of low RE and low ME. The remaining third (58 cases, 29%) showed a good PE almost equally divided between good ME and good RE (34 cases, 17%) and low RE compensated by a high ME (24 cases, 12%). Finally, only 3 cases (2%) presented a high PE.

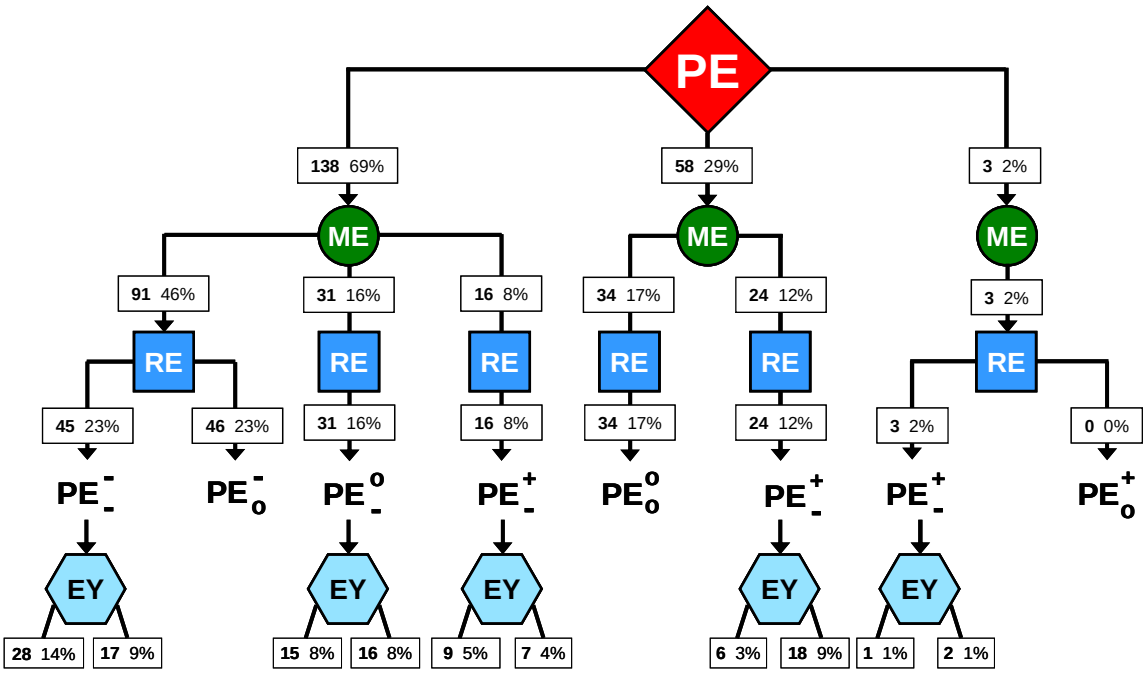

Figure 3 : general distribution of matrix effects.

It is interesting to note that plasma samples were mainly found in PE^{-}_{-} and PE^{0}_{-} situation, suggesting a more complex extraction than urine (Figure 4). However, when plasma extraction is well achieved, good ME are often encountered (PE^{0}_{0} and PE^{0}_{-}). On the other hand, urine samples dominate PE^{-}_{0} cases, demonstrating that urine endogenous compounds lead to important signal suppressions in LC-ESI-MS even after solid phase extraction. The remaining urine samples are equally distributed between the remaining low and good PE cases. Finally, high PE cases were almost not represented with this set of data, probably due to the use of ESI, known to mainly provide signal suppression rather than signal enhancement.

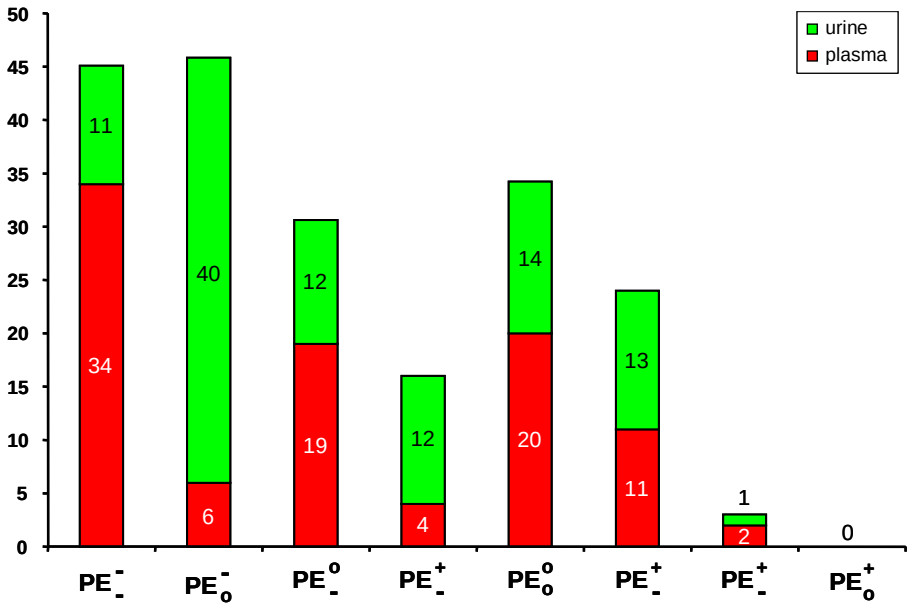

Figure 4 : matrix distribution among the eight cases.

In a general point of view (Figure 5a), no influence of the biological matrices was observed for only 17% (34) of the tested analytes. 39 % were influenced by only one parameter, 16% of the compounds (31) due to extraction issues (i.e. SPE) and 23% (46) through detector signal alterations. Almost half of the investigated set, 44% (88), suffered form the presence of endogenous compounds on both SPE and LC-MS data, demonstrating the great impact of the biological matrix toward the analytical process. Regarding ME (Figure 5b), about 67% of the compounds exhibited signal alterations (134 cases), 45% presenting signal suppression (91 cases) while the remaining 22% was concerned by signal enhancement (43 cases). As previously mentioned, signal suppressions were expected to be more abundant, since ESI was used. Finally, RE distribution (Figure 5c) was quite equilibrated, since 119 cases (60%) showed a low RE equally divided into pure low RE (60 cases, 30%) and low RE emphasized by a low M (59 cases, 30%). The remaining 40% (80 cases) did not present significant extraction problems.

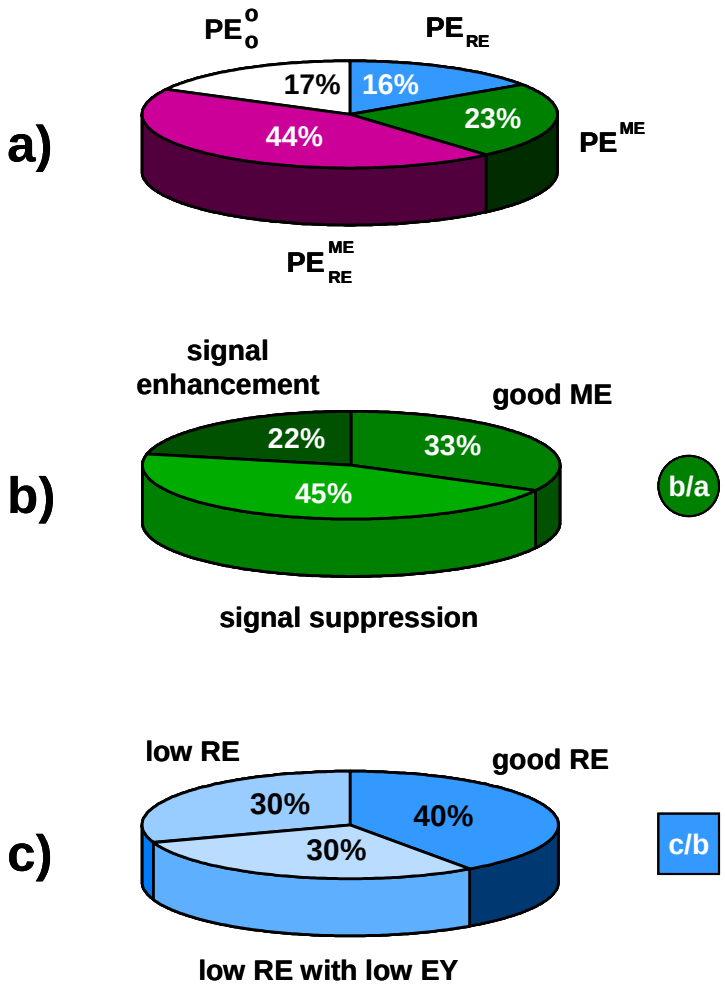

Figure 5 : a) Relative distribution of ME and RE on the whole set of data. PE^{0}_{0} indicates cases with good ME and RE, PE^{ME} is related to cases with low or high ME, PE_{RE} refers to cases with low RE and PE^{ME}_{RE} is related to cases with low or high ME and low RE. b) relative distribution of low, good and high ME. c) relative distribution of low and good RE.

3.3 Corrective actions

Matrix effects do not necessarily need to be lowered or eliminated, but identified and quantified. Indeed, the 2007 Washington workshop/conference report fixes an acceptable limit for matrix effects variability at 15% for six individual batches of the matrix [25]. When variability is higher than 15%, modifications should be operated on the concerned step(s) to reduce the influence of the matrix. Regarding signal alterations during the LC-MS analysis, the use of a deuterated internal standard (IS) is recommended. Indeed, as stated in the 2007 Washington workshop/conference report: "*Stable isotope – labelled IS minimizes the influence of matrix effects most effectively since the matrix effects observed for stable isotope - labeled IS are generally similar to those observed for the matching analyte*". If a deuterated IS is not available, an "*Analog IS may also compensate for matrix effects; however, the stable isotope - labeled internal standards are most effective and should be used whenever possible*" [25].

Chromatographic conditions can also be improved to bring the analyte out from the matrix effect window using the procedure proposed by Bonfiglio *et al.* [22]. This method allows directly identifying the chromatographic region experiencing matrix effects. When endogenous compounds prevent a satisfying extraction of the analytes with the selected procedure, protocol (re-)optimization could be reconsidered. In this regard, a sample pre-treatment (e.g. protein precipitation) can be of great help to remove the major part of endogenous compounds prior to a dedicated extraction [18].

Finally, the use of an alternative ionization source can strongly reduce matrix effects problems. Indeed, APCI and APPI have been shown to be less prone to signal alterations with MS detection [11;13;19;26]. In spite of their lower ionization yield of polar compounds, background noise is less intense, possibly leading to similar or higher signal to noise ratios. Finally, the use of direct-electron ionization (direct-EI) has also been recently proposed to overcome matrix effects encountered with MS detection for the analysis of pharmaceutical compounds in biological and environmental samples [27].

4. Conclusion

An overall classification of potential matrix effects was proposed in the case of a sample preparation followed by a LC-MS analysis. A total of eight different cases were evidenced.
Based on this classification, a schema was suggested for the attribution of the matrix effect typology. Only four samples (aqueous and matrix samples fortified prior or after sample preparation) were required, allowing the calculation of four ratios, namely PE, ME, RE, and EY. In order to illustrate the proposed method, a total of a hundred and ninety-nine analytes were used as model compounds to evaluate three different SPE and LC-MS protocols. All compounds were attributed to one of the eight possibilities. Eighty-three percent of the investigated molecules underwent matrix effects, the major part suffering from the influence of endogenous compounds during both SPE and LC-MS steps.

References

[1] W.M.A.Niessen, J. Chromatogr. A 794 (1998) 407.

[2] W.M.A.Niessen, A.P.Tinke, J. Chromatogr. A 703 (1995) 37.

[3] H.H.Maurer, J. Chromatogr. B 713 (1998) 3.

[4] P.Marquet, G.Lachatre, J. Chromatogr. B 733 (1999) 93.

[5] C.K.Lim, G.Lord, Biol. Pharm. Bull. 25 (2002) 547.

[6] J.Abian, J. Mass Spectrom. 34 (1999) 157.

[7] S.Souverain, S.Rudaz, J.L.Veuthey, J. Chromatogr. A 1058 (2004) 61.

[8] S.Souverain, S.Rudaz, J.-L.Veuthey, J. Pharm. Biomed. Anal. 35 (2004) 913.

[9] Y.Hsieh, M.Chintala, H.Mei, J.Agans, J.M.Brisson, K.Ng, W.A.Korfmacher, Rapid Commun. Mass Spectrom. 15 (2001) 2481.

[10] J.Schuhmacher, D.Zimmer, F.Tesche, V.Pickard, Rapid Commun. Mass Spectrom. 17 (2003) 1950.

[11] H.Mei, Y.Hsieh, C.Nardo, X.Xu, S.Wang, K.Ng, W.A.Korfmacher, Rapid Commun. Mass Spectrom. 17 (2003) 97.

[12] M.W.J.van Hout, H.A.G.Niederlander, R.A.de Zeeuw, G.J.de Jong, Rapid Commun. Mass Spectrom. 17 (2003) 245.

[13] P.R.Tiller, L.A.Romanyshyn, Rapid Commun. Mass Spectrom. 16 (2001) 92.

[14] L.E.Sojo, G.Lum, P.Chee, Analyst 128 (2003) 51.

[15] R.Dams, M.A.Huestis, W.E.Lambert, C.M.Murphy, J. Am. Soc. Mass Spectrom. 14 (2003) 1290.

[16] C.R.Mallet, Z.Lu, J.R.Mazzeo, Rapid Commun. Mass Spectrom. 18 (2004) 49.

[17] B.K.Choi, D.M.Hercules, A.I.Gusev, J. Chromatogr. A 907 (2001) 337.

[18] I.Marchi, S.Rudaz, M.Selman, J.L.Veuthey, Journal of Chromatography, B 845 (2007) 244.

[19] B.K.Matuszewski, M.L.Constanzer, C.M.Chavez-Eng, Anal. Chem. 70 (1998) 882.

[20] J.Henion, E.Brewer, G.Rule, Anal. Chem. 70 (1998) 650A.

[21] C.Polson, P.Sarkar, B.Incledon, V.Raguvaran, R.Grant, J. Chromatogr. B 785 (2003) 263.

[22] R.Bonfiglio, R.C.King, T.V.Olah, K.Merkle, Rapid Commun. Mass Spectrom. 13 (1999) 1175.

[23] B.K.Matuszewski, M.L.Constanzer, C.M.Chavez-Eng, Anal. Chem. 75 (2003) 3019.

[24] Food and Drug Administration, *Guidance for Industry, Bioanalytical Method Validation*, US Department of Health and Human Services, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER), 2001

[25] C.T.Viswanathan, S.Bansal, B.Booth, A.J.DeStefano, M.J.Rose, J.Sailstad, V.P.Shah, J.P.Skelly, P.G.Swann, R.Weiner, Pharm. Res. 24 (2007) 1962.

[26] Y.Hsieh, G.Wang, Y.Wang, S.Chackalamannil, J.Brisson, K.Ng, W.A.Korfmacher, Rapid Commun. Mass Spectrom. 16 (2002) 944.

[27] A.Cappiello, G.Famiglini, P.Palma, E.Pierini, V.Termopoli, H.Trufelli, Anal. Chem. 80 (2008) 9343.

Article V

Development and validation of a liquid chromatography-atmospheric pressure photoionization-mass spectrometry method for the quantification of alprazolam, flunitrazepam, and their main metabolites in haemolysed blood

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Journal of Chromatography B (*In press*, doi:10.1016/j.jchromb.2008.12.002)

ABSTRACT

A LC-APPI-MS method was developed and validated for the detection of alprazolam, flunitrazepam and their major metabolites in haemolysed blood. Samples were diluted with water (2:1) and extracted with a hydrophobic-lipophilic balanced copolymer. The method was fully validated according to ICH guidelines and SFSTP protocols. Deuterated internal standards of both parent drugs were used and good quantitative performance was achieved in terms of trueness and precision (repeatability and intermediate precision) since accuracy profiles were achieved within the acceptance limits (± 30% for biological samples). The LC-APPI-MS method was linear over the concentration range of 1-1000 and 3-1000 $ng \cdot mL^{-1}$, for alprazolam and flunitrazepam, respectively. Lower limits of quantification as low as 1 ng mL^{-1} in haemolysed blood were reached and the method was successfully applied to the quantification of alprazolam, flunitrazepam and their major metabolites in real toxicological samples.

Keywords : alprazolam, flunitrazepam, benzodiazepines, haemolysed blood, SPE, APPI, LC-APPI-MS, validation

1. Introduction

Benzodiazepines (BZD) are molecules used as psychotherapeutics that have a potent central nervous system effect. They are mainly used as tranquillizers, sedatives, anticonvulsants, and hypnotics [1,2] for the treatment of anxiety, sleep disturbances, or epilepsy [3]. They are widely consumed and their psychotropic effects often lead to behavioural disorders [4,5], dependence [6], or death by asphyxia [7]. Numerous methods for the analysis of BZD in biological matrices have already been described in the literature, mainly reporting the use of gas chromatography-mass spectrometry (GC-MS) [8-10] and liquid chromatography-mass spectrometry (LC-MS) [11-14]. Other separation techniques, such as capillary electrophoresis (CE) [15,16], micellar electrokinetic chromatography (MEKC) [17,18] and capillary electrochromatography (CEC) [17], have also been reported.

Concerning LC-MS, most applications have been conducted with electrospray ionization (ESI) [4,12,19], while atmospheric pressure chemical ionization (APCI) [20,21], fast atom bombardment (FAB) [22], matrix-assisted laser desorption ionization (MALDI) [23], and atmospheric pressure thermodesorption surface ionization (APTDSI) [24] techniques have been rarely used. To the best of our knowledge, no LC-MS methods have been published with atmospheric pressure photoionization (APPI) for BZD analysis in biological samples. However, the APPI source presents various advantages over ESI, such as a lower sensitivity to signal alterations with biological samples [25-30] and a larger linear dynamic range [31,32]. The latter is particularly well adapted to BZD analysis because of their wide therapeutic and toxicity windows [33,34]. Most studies have reported BZD analysis in urine [35-38], but other body fluids have also been investigated, such as blood [10,39,40], hair [11,41], and saliva [42]. Because direct injection of such samples in LC-MS systems presents difficulties, a sample preparation is necessary. Among the available techniques, liquid-liquid extraction (LLE) [10,43,44] and solid-phase extraction (SPE) [12,14,37] are the most employed, whereas the use of solid-phase micro-extraction (SPME) [4,39] and supercritical fluid extraction (SFE) [45] have been less reported. SPE presents the advantage of a fast procedure, solvents compatibility with LC mobile phases and ease of automation over LLE.

The aim of this study was to develop and validate a method to quantify the commonly consumed BZD alprazolam and flunitrazepam, as well as their respective major metabolite, namely α-hydroxyalprazolam and 7-aminoflunitrazepam, in haemolysed blood samples, an important matrix for toxicological or forensic issues. Method selectivity was evaluated with various other BZD to avoid co-medication issues. A simple and fast SPE procedure was implemented, followed by a selective LC separation in the isocratic mode coupled to a sensitive APPI-MS detector. Quantitative performance was assessed according to the international conference on harmonization (ICH) guidelines as well as recommendations from the “société française des sciences et techniques pharmaceutiques” (SFSTP) [46-48] including the concept of total error. Various criteria, namely trueness, precision, accuracy and limit of quantification (LOQ) were used to evaluate quantitative performance. Deuterated internal standards (IS) were used to compensate for the overall method variability, including extraction and ionization variations. The fully validated method was applied to the quantification of alprazolam and flunitrazepam in toxicological cases.

2. Experimental

2.1 Chemicals

Stock solutions at 1000 µg·mL^{-1} in methanol (MeOH) of flunitrazepam (FLZ), flunitrazepam-D_7 (FLZ-D_7) 100.00% isotopic purity, 7-aminoflunitrazepam (AFLZ), alprazolam (ALZ), alprazolam-D_5 (ALZ-D_5) 99.91% isotopic purity, and α-hydroxyalprazolam (HALZ) were purchased from Cerilliant (Austin, Texas, USA) and stock solutions at 1000 µg·mL^{-1} in MeOH of lorazepam (LRZ), clonazepam (CLZ), nitrazepam (NTZ), and oxazepam (OXZ) were provided by Lipomed (Arlesheim, Switzerland). Chemical structures of the analytes are reported in Figure 1. Acetonitrile (ACN) and MeOH were purchased from Panreac (Barcelona, Spain). Acetic acid 99.8%, ammonium hydroxide 25%, acetone, and toluene were obtained from Fluka. Water was provided by a Milli-Q Gradient A10 water purifier system from Millipore (Bedford, MA, USA). All chemicals were of the highest purity grade commercially available and all reagents used were of HPLC grade. Human blank whole blood and real cases were obtained from the Institut Universitaire de Médecine Légale (IUML, Geneva, Switzerland). They were stored at -22℃ and defrosted at room temperature for 30 minutes before use. Because freezing and thawing cause hemolysis of human erythrocytes [49], they were considered as haemolysed blood samples.

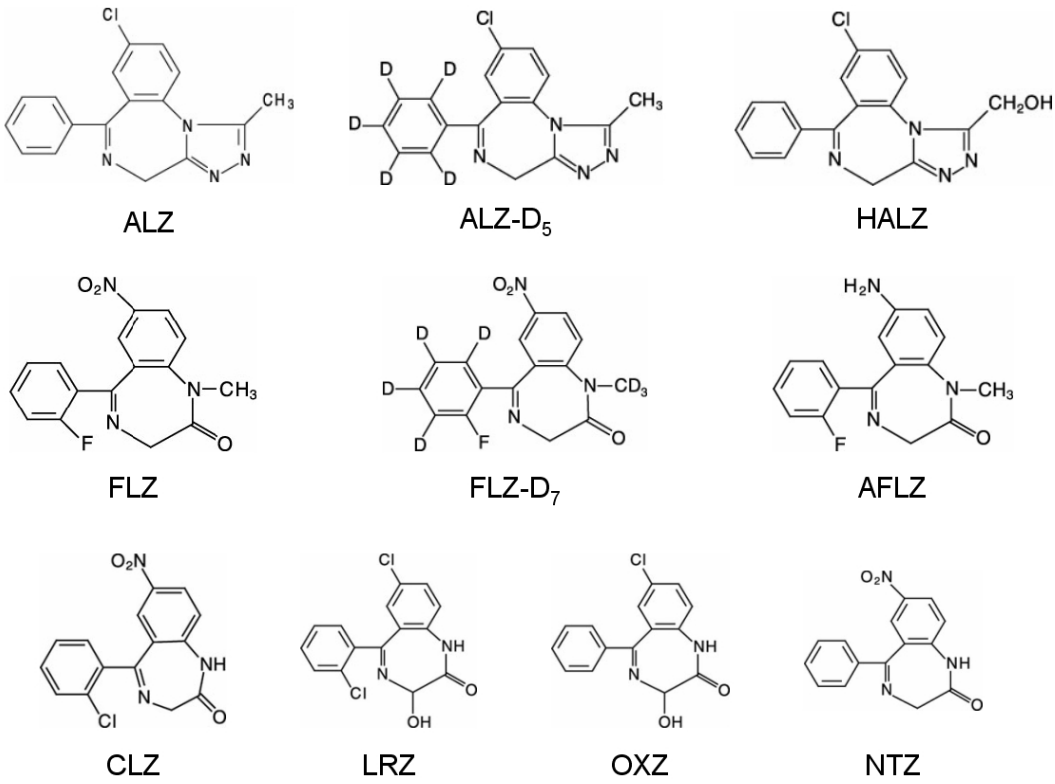

Figure 1 : Chemical structures of investigated BZD.

2.2 Sample preparation

2.2.1 SPE

Haemolysed blood samples were centrifuged at 8000 *x g* for 10 minutes and 1000 µL of water was added to 500 µL of the supernatant. After vortex-mixing, the whole sample (1500 µL) was extracted by SPE on an Oasis HLB 96-well plate (10 mg sorbent) from Waters (MA, USA). Each well was conditioned with 500 µL of MeOH and equilibrated with 500 µL of water. One thousand five hundred microliters of the sample was loaded and washed with a mixture of water-MeOH (70:30, v/v). Elution was carried out with 250 µL of MeOH and the eluate was directly transferred into the injection vial.

2.2.2 Evaluation of process efficiency and matrix effect

Recoveries (*i.e.*, process efficiency) were estimated according to the methodology developed by Matuszewski *et al.* [50]. A first set of three standards was prepared using neat solutions of BZD in MeOH at three concentrations, namely LOQ, 50% and 100% of the studied range (Table 1). The samples were prepared by diluting appropriate volumes of each BZD stock solution with MeOH (total volume of 250 µL). After mixing, the solutions were transferred into injection vials and directly injected into the LC-APPI-MS system. A second set of three standards was prepared in haemolysed blood originating from six different sources, pooled, and spiked before SPE. The samples were prepared by diluting appropriate volumes of each BZD stock solution with haemolysed blood (total volume of 500 µL). After mixing, the solutions were subjected to the above-mentioned SPE procedure. Spiked amounts were calculated to obtain the same concentrations of BZD after SPE than in samples from the first set. The mean recovery, as well as associated RSD, were determined for each BZD at each concentration by the ratio of peak areas obtained in the second set to those in the first set (Table 3).
Matrix effect on LC-APPI-MS was investigated using a post-column infusion system according to Bonfiglio *et al.* [51]. 5 µL of mobile phase, water, and blank haemolysed blood extracted by the above-mentioned SPE procedure was injected in the LC-APPI-MS system, while a solution containing all BZD in the mobile phase at 10 ng mL^{-1} was infused post column at a flow rate of 2 µL min^{-1} by a Harvard 11 Plus Single Syringe pump (South Natick, MA, USA). Effects associated to the elution of endogenous compounds on the analytes signal were assessed by comparing the MS response obtained with the injection of extracted blank blood to that of the mobile phase.

2.2.3 Samples used for calibration

Calibration standards (CS) were prepared through an independent method from blank haemolysed blood spiked with known concentrations of analytes and their respective deuterated IS at a fixed concentration. Three concentration levels were selected (k=3), corresponding to low (estimated

LOQ), medium (50%) and high (100%) concentrations. The investigated ranges of each BZD are summarized in Table 1. The CS were replicated twice (n=2) on three different series (j=3).

Table 1 : Calibration standard (CS, k=3) and quality control (QC) sample (k=4) concentrations ($ng \cdot mL^{-1}$) used for validation of alprazolam (ALZ), flunitrazepam (FLZ) and their respective major metabolite, α-hydroxyalprazolam (HALZ) and 7-aminoflunitrazepam (AFLZ).

CS	QC	ALZ	HALZ	FLZ	AFLZ
LOQ	LOQ	1	13	3	2
	4*LOQ	4	52	12	8
50%	50%	500	500	500	500
100%	100%	1000	1000	1000	1000

2.2.4 Samples used for validation

Validation standards or quality control samples (QC) were prepared through an independent method from blank haemolysed blood spiked with known concentrations of analytes and their respective deuterated IS at a fixed concentration. Concentrations of each analyte are summarized in Table 1. Four concentration levels were selected (k=4) and QC were replicated four times (n=4) on three different series (j=3).

2.2.5 Application to biological samples

Two frozen blood samples (011 987 and 180 07) were provided from the IUML (Geneva, Switzerland). Sample 011 987 contained FLZ and sample 180 07 contained ALZ, both at concentrations lower than 100 $ng \cdot mL^{-1}$. They were defrosted under agitation at room temperature for 30 minutes. Both samples were centrifuged at 8000 *x g* for 10 minutes. One thousand microliters of an aqueous solution of FLZ-D_7 at 180 $\mu g \cdot mL^{-1}$ was added to 500 µL of the supernatant of sample 011 987. One thousand microliters of an aqueous solution of ALZ-D_5 at 110 $\mu g \cdot mL^{-1}$ was added to 500 µL of the supernatant of sample 180 07. Each sample was then extracted by SPE as described in paragraph 2.2.1. Based on the available volumes, sample 011 987 was extracted twice (N=2) and sample 180 07 three times (N=3).

2.3 Instrumentation

All experiments were performed on an Agilent Series 1100 LC system (Agilent Technologies, Waldbronn, Germany) equipped with an autosampler and a binary pump. Five microliters of the sample was injected on n XBridge Shield 100 x 2.1 mm, 3.5 µm analytical column from Waters (MA, USA). The mobile phase, acetate buffer 20 mM pH 5-ACN (67:33, v/v), was delivered in the isocratic

mode at 200 µL·min^{-1}. The LC system was coupled to an Agilent Series 1100 MSD single quadrupole equipped with an orthogonal APPI source (PhotoMate). Nitrogen was used as both the nebulizing (5 L·min^{-1}) and drying gas (250°C). The vaporizer temperature was set at 250°C, nebulizer pressure at 45 psig and capillary voltage at +2000 V. Post-column infusion of acetone as a dopant was achieved at 20 µL·min^{-1} by a Harvard 11 Plus Single Syringe pump (South Natick, MA, USA). Detection of protonated FLZ, AFLZ, ALZ, HALZ, LRZ, CLZ, NTZ, and OXZ was conducted in the selected ion monitoring (SIM) mode at *m/z* 314, 284, 309, 325, 321, 316, 282, and 287, respectively, with a dwell time of 71 ms. The Chemstation A.10.03 software (Agilent Technologies) was used for instrument control, data acquisition and data handling.

3. Results and discussion

The aim of this study was to develop and validate a LC-APPI-MS method for the analysis of two broadly used BZD in haemolysed blood, namely alprazolam (ALZ) and flunitrazepam (FLZ), as well as their major metabolites, α-hydroxyalprazolam (HALZ) and 7-aminoflunitrazepam (AFLZ). Metabolites quantification was carried out to establish a BZD intake even in the case of an undetectable amount of the substrate. Lorazepam (LRZ), clonazepam (CLZ), nitrazepam (NTZ), and oxazepam (OXZ) were included in the evaluation of the method selectivity to avoid quantification problems due to potential co-medication.

The set of compounds includes weak bases (ALZ, FLZ, and AFLZ, pK_a<3.1), a weak acid (LRZ, pK_a>10.8) and ampholytes with both weak functions (CLZ, NTZ, OXZ and HALZ) (Table 2). Since BZD possess broad therapeutic and toxicity windows (*e.g.,* between 5 ng·mL^{-1} and 400 ng·mL^{-1} for ALZ [34]), the analytical procedure should therefore enable quantification over a wide concentration range.

Table 2 : Studied BZD with their pKa, logD and typical blood concentrations (µg·mL^{-1}).

compound	pKa [1]		logD [1]			concentration in blood (µg·mL^{-1}) [34]		
	acidic	basic	pH 2	pH 7	pH 10	therapeutic	toxic	lethal
alprazolam	-	2.3	1.9	2.5	2.5	0.005 - 0.05	0.1 - 0.4	-
α-hydroxyalprazolam	13.1	1.2	1.6	1.7	1.7		-	
clonazepam	11.2	1.5	2.2	2.3	2.3	0.01 - 0.08	0.1	-
flunitrazepam	-	1.7	1.1	1.3	1.3	0.005 - 0.015	0.05	-
7-aminoflunitrazepam	-	3.1	-0.6	0.6	0.6		-	
lorazepam	10.8	-	2.5	2.5	2.4	0.08 - 0.25	0.3 - 0.5	-
nitrazepam	11.4	3.2	1.0	2.2	2.2	0.03 - 0.1	0.2 - 0.3	5
oxazepam	10.9	1.7	2.1	2.3	2.3	0.2 - 1.5	2	3 - 5

[1] pK_a and log D values were calculated using Advanced Chemistry Development software version 8.14 for Solaris (ACD/Labs, Toronto, Canada).

3.1 Method development

3.1.1. SPE

A hydrophilic-lipophilic sorbent (Oasis HLB) was selected. In order to determine the most suitable operating conditions, experiments were first performed on neat standard solutions spiked with all compounds and then transferred to haemolysed blood samples.

Analyte retention on the sorbent during the loading step was compared in acidic and neutral media (pH 2.5 and 7). As expected, the Oasis HLB was not able to fully retain analytes under acidic conditions, due to the lower hydrophobicity of BZD (Table 2) than at neutral pH. However, the latter allowed compound retention and was therefore selected. The washing step was then studied with mixtures of water/MeOH between 0% and 100% of MeOH. The optimal amount of MeOH was 30% for minimal compound loss at this stage. Finally, increasing volumes of MeOH (100 µL, 250 µL and 500 µL) for elution were evaluated and 250 µL was found to be optimal for a complete elution from a 10 mg sorbent cartridge.

This SPE procedure was implemented on haemolysed blood samples spiked with all analytes. Potential interference of endogenous compounds during SPE and LC-MS analysis could thus be emphasized. Haemolysed blood was first centrifuged and the supernatant diluted with two volumes of water as a sample pretreatment before SPE. As already observed elsewhere [26], dilution prior to on-line SPE removed matrix effects encountered with APPI for some analytes, whereas ESI was subjected to such effects in all cases. In this study, as an off-line SPE was operated, qualitative information on matrix effects resulting from a simple dilution of the sample prior to SPE was retrieved from a post-column infusion set-up (2.2.2) [51]. As no interferences were observed (data not shown), dilution was selected for its ease and to limit co-precipitation risks. Quantitative results on matrix effects were obtained through the application of the method described by Matuszewski *et al.* [50]. Recoveries on elution were calculated at low and high concentration levels (estimated LOQ and 100%, respectively, see Table 1) by comparing results obtained with neat standards not extracted on SPE (2.2.2). Mean recoveries were between 83% and 119% for all compounds and RSD values were lower than 10% (Table 3), confirming no matrix effects. The simple dilution of the sample prior to SPE was therefore considered as adapted to off-line SPE combined with APPI.

Table 3 : SPE elution step: mean recovery of haemolysed blood samples spiked with the studied BZD at each CS concentration level.

	Mean recovery (%)		RSD (%)	
	at LOQ	at 100%	at LOQ	at 100%
alprazolam	102.9	116.0	3.2	9.6
α-hydroxyalprazolam	95.1	106.7	6.3	6.6
flunitrazepam	96.8	106.2	2.4	3.0
7-aminoflunitrazepam	107.7	119.2	6.0	6.0
lorazepam	99.9	113.8	6.2	7.6
clonazepam	86.4	96.0	3.3	2.7
nitrazepam	84.4	96.4	5.5	3.3
oxazepam	87.2	92.4	6.5	1.1

3.1.2. LC-APPI-MS

Since there were two pairs of isobaric compounds (*e.g.,* FLZ/ALZ-D_5, *m/z* 314 and LRZ/FLZ-D_7, *m/z* 321), a chromatographic separation with sufficient resolution was necessary. Three columns were compared (Waters XBridge 100 x 2.1 mm, 3.5 µm, Waters XBridge Shield 100 x 2.1 mm, 3.5 µm and Thermo Hypersil Gold 100 x 2.1 mm, 5 µm) and mobile phases at various pH and compositions were tested. The optimal chromatographic conditions for BZD separation were found using HPLC modeling software (Osiris 4.1.1.2, Datalys, Grenoble, France) according to a procedure described in the literature [52]. The XBridge Shield presented the best compromise between resolution of both isobaric couples and analysis time, with a mobile phase made of acetate buffer 20 mM pH 5-ACN (67:33, v/v). The mobile phase was delivered at 200 $\mu L \cdot min^{-1}$ to fulfill APPI requirements regarding the maximal affordable flow rate [53]. Under these chromatographic conditions, complete separation (Rs>1.5) of isobaric compounds was achieved, with a total analysis time of 10 minutes (Figure 2). Isotopic abundance of ^{35}Cl / ^{37}Cl occurred in most investigated BZD and accounted for small peaks on relatively close (in term of *m/z*) extracted chromatograms. For instance, ALZ-D_5, (*m/z* 314) presents a small *m/z* difference with CLZ (*m/z* 316). Therefore, a small peak corresponding to ALZ-D_5 was found on CLZ chromatogram around 5.5 min.

Since HLB elution required pure MeOH, chromatographic performance might be affected by the direct injection of the organic fraction [54]. Hence, evaporation and reconstitution in water was considered. The influence of the injection solvent on the chromatographic separation was evaluated by the injection of standard solutions diluted in increasing proportions of MeOH. Pure MeOH did not alter the resolution, probably due to the low injected volume (5 µL) and to the retention factors between 2 and 8.

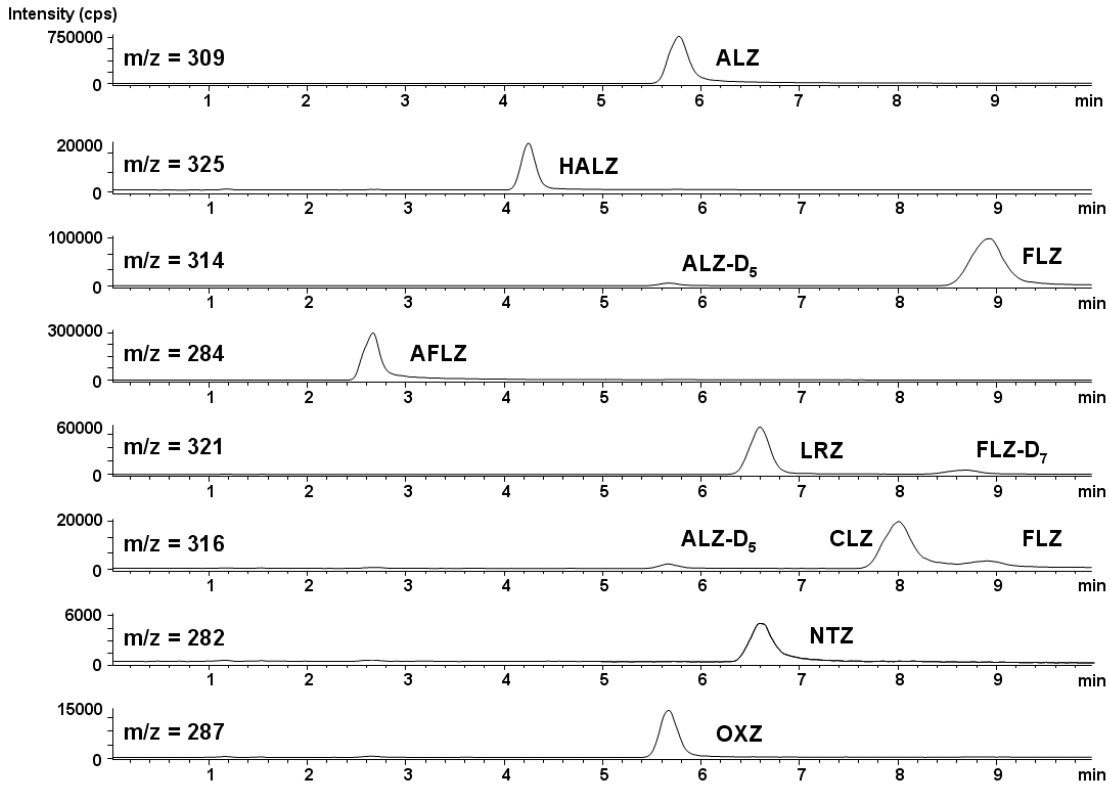

Figure 2 : Chromatograms obtained by the injection of studied BZD diluted in MeOH at 250 ng·mL^{-1}.

Source parameters were optimized with standard solutions and the best compromise for all compounds was selected. It has to be noted that the selected capillary voltage in the APPI was quite high (+ 2 kV) compared to standard settings. However, no ionization was observed by switching off the lamp, indicating that ionization under these conditions was exclusively due to photoionization and not ESI-like mechanisms. BZD photoionization was evaluated with and without dopant, since the latter could significantly improve the ionization process [55-58]. The two most common dopants, toluene and acetone [56], were investigated. Both were added at 10% of the mobile phase flow rate (*i.e.,* 20 µL·min^{-1}) since ionization efficiency reaches a maximum at this proportion [55]. This was done through post-column infusion with a tee placed between the column and the detector, to prevent from chromatographic and/or solubility issues compared to direct addition in the mobile phase. The use of toluene was deleterious compared to the no dopant situation, while acetone significantly improved signals (factor 1.5 on ALZ, HALZ, FLZ and AFLZ, n=6) and was therefore selected (data not shown). It has to be noted that the use of acetone as a dopant in APPI was generally found in the literature to be less efficient than toluene regarding proton transfer. The latter was also proved to promote charge transfer, a mechanism unachievable with acetone [57,59]. However, in the case presented here, pseudo-molecular ions were mainly observed over molecular ions in all cases when acetone was used rather than toluene, as already observed in a previous study [26].

Finally, sensitivity was compared in ESI and APPI with standard solutions of all used benzodiazepines and was found better in APPI by a factor 3 with ALZ and a factor 2 with FLZ. The

use of APCI could be a good alternative to APPI for the ionization of BZD with chromatography performed at high flow rates (> 1 ml·min^{-1}) due to its ionization mechanism [55].

3.2 Quantitative performance

In order to compensate for the overall method variability, including extraction and ionization variations, deuterated IS of ALZ and FLZ were employed. Quantitative determinations of ALZ, HALZ, FLZ and AFLZ are presented.

3.2.1 Selectivity

Because endogenous compounds might still be present after solid phase extraction and induce ionization alterations, potential matrix effects were evaluated, as mentioned in paragraph 3.1.1. Since no interference was observed, method selectivity was further investigated by comparing chromatograms obtained by the injection of blank haemolysed blood (CAL 00), blank haemolysed blood spiked with IS (CAL 0, ALZ-D_5 and FLZ-D_7 at 220 ng·mL^{-1} and 360 ng·mL^{-1}, respectively), and a CS at the estimated LOQ. As illustrated in Figure 3, no interferences were observed at retention times corresponding to analytes of interest and IS, although six independent sources of haemolysed blood were tested. It has to be noted that the peak appearing at eight minutes in Figures 3A and B corresponded to FLZ, while the peak appearing at six minutes in Figures 3C corresponded to ALZ-D_5. Both compounds are isobaric (*m/z* 314) and could not be resolved with a single quadrupole mass spectrometer. Figures 3C and D also revealed another peak at seven minutes, attributable to LRZ that presents the same *m/z* value (321) to that of FLZ-D_7. A selective LC separation was thus mandatory prior to MS detection with a single quadrupole analyzer to prevent from this issue.

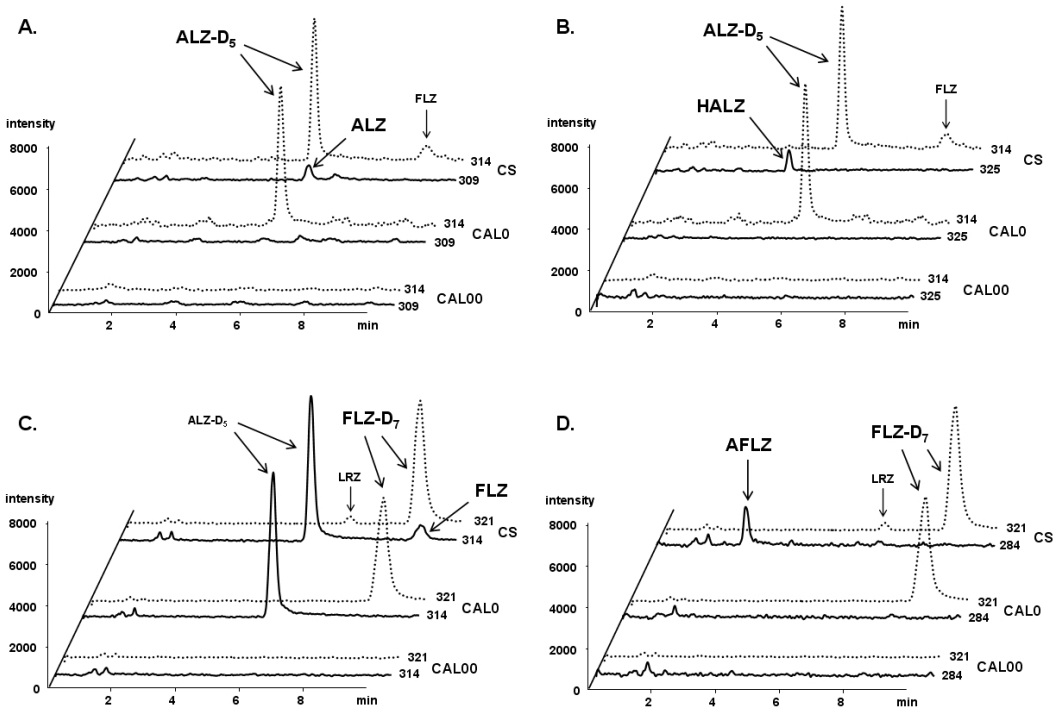

Figure 3 : A) Typical chromatograms of ALZ obtained by the injection of blank haemolysed blood (CAL 00), blank haemolysed blood spiked with ALZ-D_5 (CAL 0) at 220 ng·mL^{-1} and a calibration standard (CS) containing ALZ at 1 ng·mL^{-1}, B) Typical chromatograms of HALZ obtained by the injection of blank haemolysed blood (CAL 00), blank haemolysed blood spiked with ALZ-D_5 (CAL 0) at 220 ng·mL^{-1} and a calibration standard (CS) containing HALZ at 13 ng·mL^{-1}, C) Typical chromatograms of FLZ obtained by the injection of blank haemolysed blood (CAL 00), blank haemolysed blood spiked with FLZ-D_7 (CAL 0) at 360 ng·mL^{-1} and a calibration standard (CS) containing FLZ at 3 ng·mL^{-1}, and D) Typical chromatograms of AFLZ obtained by the injection of blank haemolysed blood (CAL 00), blank haemolysed blood spiked with FLZ-D_7 (CAL 0) at 360 ng·mL^{-1} and a calibration standard (CS) containing AFLZ at 2 ng·mL^{-1}. XIC of ALZ (*m/z*=309), HALZ (*m/z*=325), ALZ-D_5 (*m/z*=314), FLZ (*m/z*=314), AFLZ (*m/z*=284), and FLZ-D_7 (*m/z*=321).

3.2.2 Validation

Quantitative performance was evaluated according to SFSTP validation guidelines on three separate series (j=3). According to SFSTP 2003 recommendations [47], validation protocol V5 was followed. The latter recommends three concentration levels (k=3) with two repetitions (n=2) for CS. To improve accuracy profiles, four concentration levels (k=4) with four repetitions (n=4) for QC, both prepared in the biological matrix, were selected. Trueness and precision were estimated for each concentration level after the selection of the most suitable response function. Trueness corresponds to the difference between the true value and the mean recalculated concentration of QC (experimental value). Trueness was expressed in terms of relative bias (%) for each level of concentration and for every compound. Precision corresponds to the dispersion level among a series of measurements from multiple samplings. In this study, precision was estimated with variances of repeatability (s^2_r) and intermediate precision (s^2_R), calculated on the estimated concentrations as described in [60] and finally expressed as RSD. Accuracy corresponds to the total error including systematic (trueness) and

random (precision) errors. For the construction of accuracy profiles used to evaluate the total error of the method, SFSTP 1997 [46] recommendations were followed. Confidence intervals were calculated for each compound with fixed degrees of freedom (df=j·(n-1)) at a unilateral level of risk α=10%. These confidence intervals gave the lower and upper confidence limits associated to the experimental value.

3.2.2.1 Regression model selection

Since the calibration curve affects validation results, various regression models were compared. Ordinary least squares (OLS), OLS forced through zero, external standard at the highest CS level, OLS after logarithmic transformation of both concentration (x) and response (y), OLS after square root transformation of both concentration (x) and response (y) and weighted least squares (WLS) with two weighting factors (1/x and $1/x^2$) were tested for each compound. QC concentrations were back calculated *via* slope and intercept from each tested response function to determine the mean bias and the confidence limits (lower and upper). The acceptance limit for accuracy profiles was fixed at ±30%, in accordance with the most recent regulatory recommendations [61]. These profiles were used as a decision tool to estimate the method ability to quantify samples with an accepted risk (α=10%). Figure 4 shows selected accuracy profiles for ALZ, HALZ, FLZ and AFLZ.

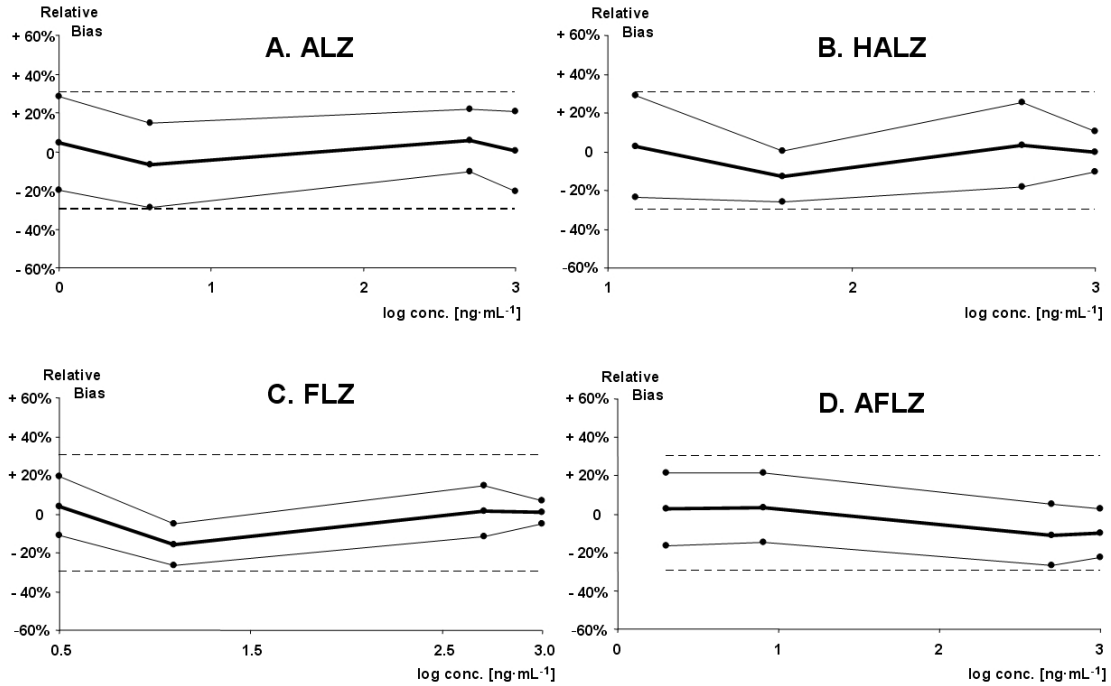

Figure 4 : Accuracy profiles expressed as confidence intervals for A) ALZ, B) HALZ, C) FLZ, and D) AFLZ.

The developed method was considered accurate over the investigated concentration ranges for every compound since the lower and upper confidence limits did not exceed the acceptance limits (±30%) (Table 4). The most appropriate regression model covering the whole concentration range was then selected. The most suitable calibration model was the WLS with $1/x^2$ as a weighting factor for ALZ, HALZ and FLZ, whereas for AFLZ the best model was the linear regression after logarithmic transformation.

Table 4 : Validation results for A) ALZ, B) HALZ, C) FLZ, and D) AFLZ (j=3; k=4; n=4).

A)	Validation criterion	alprazolam
	Trueness	
	Relative bias (%)	
	1 (ng·mL-1)	4.2
	4 (ng·mL-1)	-7.1
	500 (ng·mL-1)	5.6
	1000 (ng·mL-1)	0.1
	Precision	
	Repeatability / Intermediate precision (RSD, %)	
	1 (ng·mL-1)	9.4 / 13.2
	4 (ng·mL-1)	4.8 / 12.0
	500 (ng·mL-1)	8.3 / 8.8
	1000 (ng·mL-1)	4.5 / 11.2
	Accuracy	
	Lower / upper limits of the total error (%)	
	1 (ng·mL-1)	-20.1 / 28.5
	4 (ng·mL-1)	-29.2 / 14.9
	500 (ng·mL-1)	-10.6 / 21.8
	1000 (ng·mL-1)	-20.5 / 20.7
	Linearity	
	Range (ng·mL-1)	[1;1000]
	Slope	1.006
	Intercept	4.8596
	R2	0.9992
	LLOQ (ng·mL-1)	1

B)	Validation criterion	α-hydroxyalprazolam
	Trueness	
	Relative bias (%)	
	13 (ng·mL-1)	2.9
	52 (ng·mL-1)	-12.8
	500 (ng·mL-1)	3.6
	1000 (ng·mL-1)	-0.2
	Precision	
	Repeatability / Intermediate precision (RSD, %)	
	13 (ng·mL-1)	10.1 / 14.3
	52 (ng·mL-1)	3.9 / 7.1
	500 (ng·mL-1)	10.0 / 11.8
	1000 (ng·mL-1)	4.1 / 5.8
	Accuracy	
	Lower / upper limits of the total error (%)	
	13 (ng·mL-1)	-23.3 / 29.0
	52 (ng·mL-1)	-25.7 / 0.2
	500 (ng·mL-1)	-18.1 / 25.2
	1000 (ng·mL-1)	-10.7 / 10.4
	Linearity	
	Range (ng·mL-1)	[13;1000]
	Slope	1.005
	Intercept	0.585
	R2	1.000
	LLOQ (ng·mL-1)	13

C)

Validation criterion	flunitrazepam
Trueness	
Relative bias (%)	
3 (ng·mL-1)	4.1
12 (ng·mL-1)	-15.7
500 (ng·mL-1)	1.4
1000 (ng·mL-1)	0.8
Precision	
Repeatability / Intermediate precision (RSD, %)	
3 (ng·mL-1)	5.8 / 8.2
12 (ng·mL-1)	4.4 / 5.9
500 (ng·mL-1)	7.1 / 7.1
1000 (ng·mL-1)	3.3 / 3.3
Accuracy	
Lower / upper limits of the total error (%)	
3 (ng·mL-1)	-11.0 / 19.2
12 (ng·mL-1)	-26.5 / -4.9
500 (ng·mL-1)	-11.6 / 14.4
1000 (ng·mL-1)	-5.2 / 6.8
Linearity	
Range (ng·mL-1)	[3;1000]
Slope	1.010
Intercept	-0.3241
R2	1.0000
LLOQ (ng·mL-1)	3

D)

Validation criterion	7-aminoflunitrazepam
Trueness	
Relative bias (%)	
2 (ng·mL-1)	2.7
8 (ng·mL-1)	3.2
500 (ng·mL-1)	-11.0
1000 (ng·mL-1)	-9.9
Precision	
Repeatability / Intermediate precision (RSD, %)	
2 (ng·mL-1)	10.0 / 10.4
8 (ng·mL-1)	8.1 / 9.8
500 (ng·mL-1)	7.4 / 8.7
1000 (ng·mL-1)	4.9 / 6.8
Accuracy	
Lower / upper limits of the total error (%)	
2 (ng·mL-1)	-16.4 / 21.7
8 (ng·mL-1)	-14.7 / 21.1
500 (ng·mL-1)	-26.8 / 4.9
1000 (ng·mL-1)	-22.3 / 2.6
Linearity	
Range (ng·mL-1)	[2;1000]
Slope	0.900
Intercept	-0.4183
R2	1.0000
LLOQ (ng·mL-1)	2

3.2.2.2 Linearity

A linear method gives results directly proportional to the concentration of the analyte over the investigated range. For each compound, a linear regression model was applied to the recalculated QC concentrations *vs.* experimental concentrations. The slope, intercept and coefficient of determination were calculated for each model. In all cases, slopes and intercepts were between 0.900 – 1.010 and -0.42 – 4.86, respectively. The R^2 values were higher than 0.9992, indicating that the developed method was linear for the tested compounds.

3.2.2.3 Limit of quantification (LOQ)

The lower limit of quantification (LLOQ) defines the lowest amount of analyte that can be measured in the matrix under the experimental conditions with a defined accuracy. Since the lowest concentration levels for each compound were included in the acceptance limits, they were considered as the LLOQ (1 ng·mL^{-1}, 13 ng·mL^{-1}, 3 ng·mL^{-1} and 2 ng·mL^{-1} for ALZ, HALZ, FLZ and AFLZ, respectively). In

comparison with ESI and APCI, the LOQ achieved for ALZ and FLZ in APPI with our single quadrupole MS was analogous to those obtained in the literature in ESI with triple quadrupole MS or ion trap MS (in the 0.5 to 5.0 ng·mL^{-1} range) [11;62-66] and in APCI with single quadrupole MS or ion trap MS (in the 1.0 to 5.0 ng·mL^{-1} range) [20,44,67,68].

3.2.2.4 Trueness and precision

Trueness was acceptable in each case (threshold of ±15%) as the relative bias was lower than 12.8% except for FLZ at 12 ng·mL^{-1} (-15.7%) (Table 4). However, this higher value was compensated by good precision, keeping the total error, expressed as the confidence interval, under ±30%. Regarding precision, RSD values were satisfactory, since they were all between 3.3% and 10.1% for repeatability, and between 3.3% and 14.3% for intermediate precision. The best results were obtained with FLZ (3.3% / 7.1% and 3.3% / 8.2% for repeatability and intermediate precision, respectively).

3.2.3 Application to biological samples

The applicability of the developed method was illustrated with the analysis of two real haemolysed blood samples (180 07 and 011 987). A preliminary LC-MS screening was first performed by the Institut Universitaire de Médecine Légale which showed that sample 180 07 contained ALZ and sample 011 987 contained AFLZ. The method was applied to both samples and quantification of each compound was based on a calibration curve obtained on the same day. CS with the same concentrations as those used during the validation process (k=3) were injected twice (n=2) and the same data treatment was used (WLS with $1/x^2$ as weighting factor for ALZ, HALZ and FLZ and OLS after logarithmic transformation for AFLZ). The confidence interval was calculated with Formula 1.

$$\bar{x} = t_{df,\alpha}\sqrt{\frac{s_r^2}{N} + s_g^2} \qquad (1)$$

where $\bar{x}$ is the mean result and N is the number of analyses. The $t_{df,\alpha}$ (Student constant depending on the degrees of freedom (df) and on the level of significance α), s^2_r and s^2_g (intra and inter-series variances) values were determined during validation *via* the regular ANOVA-based variance decomposition. Since an important part of the overall variability was attributed to repeatability, samples were analyzed twice (sample 011 987) and thrice (sample 180 07) to decrease the confidence interval. In routine analysis, this can be carried out in a single measurement according to the obtained validation results. Chromatograms of both samples are given in Figure 5.

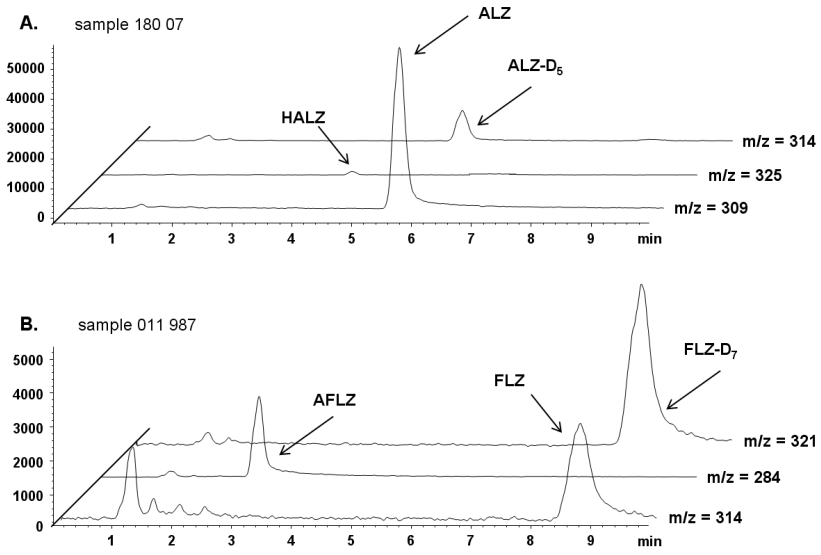

Figure 5 : Chromatograms obtained from the injection of toxicological samples. A) haemolysed blood sample 180 07 spiked with ALZ-D_5 at 220 ng·mL^{-1} and B) haemolysed blood sample 011 987 spiked with FLZ-D_7 at 360 ng·mL^{-1}.

A concentration of 7.6 ng·mL^{-1} ± 0.5 ng·mL^{-1} of FLZ was found in sample 011 987, but the sample also contained 27.5 ng·mL^{-1} ± 9.6 ng·mL^{-1} of AFLZ, indicating that the majority of the FLZ was metabolized (see Table 2). Concerning sample 180 07, it contained 64.6 ng·mL^{-1} ± 18.2 ng·mL^{-1} of ALZ and 24.7 ng·mL^{-1} ± 3.5 ng·mL^{-1} of HALZ, a concentration within the toxicity window considering plasma values [34]. However, no data were found in the literature to evaluate these values in haemolysed blood samples.

4. Conclusion

A method for determining alprazolam, flunitrazepam and their major metabolites in haemolysed blood was developed. The sample preparation was achieved by a fast and easy SPE without evaporation or reconstitution. A selective and sensitive LC-APPI-MS analysis was carried out in less than 10 minutes. The use of the APPI source was found to be an excellent alternative to ESI and APCI since it lead to similar sensitivities without matrix effects. A validation strategy based on accuracy profiles was applied on alprazolam, flunitrazepam and their major metabolites to demonstrate the methods ability to quantify these compounds in haemolysed blood over a wide concentration range. Selectivity, trueness, precision and the lower limit of quantification were calculated by applying an appropriate regression model and good performance was achieved (accuracy was included in the acceptance limit of ±30%). The developed technique was finally applied to rapidly quantify alprazolam, flunitrazepam and their metabolites in two toxicological samples.

Acknowledgements

The authors wish to acknowledge Dr. Christian Staub of the Institut Universitaire de Médecine Légale (Geneva) for the forensic cases and the standard solutions.

Reference list

[1] A. Bugey, C. Staub, J. Pharm. Biomed. Anal. 35 (2004) 555.

[2] A. Aresta, L. Monaci, C.G. Zambonin, J. Pharm. Biomed. Anal. 28 (2002) 965.

[3] D. Kavvadias, A.A. Abou-Mandour, F.C. Czygan, H. Beckmann, et al., Biochem. Biophys. Res. Commun. 269 (2000) 290.

[4] H. Yuan, Z.Mester, H.Lord, J.Pawliszyn, J. Anal. Toxicol. 24 (2000) 718.

[5] F. Barbone, A.D. McMahon, P.G. Davey, A.D. Morris, et al., Lancet 352 (1998) 1331.

[6] O.H. Drummer, J. Chromatogr. B 713 (1998) 201.

[7] Y. Kamijo, I. Hayashi, T. Nishikawa, K. Yoshimura, K. Soma, J. Anal. Toxicol. 29 (2005) 140.

[8] D. Blachut, M. Bykas-Strekowska, E. Taracha, B. Szukalski, Z Zagadnien Nauk Sadowych 59 (2004) 5.

[9] S. Pirnay, S. Bouchonnet, F. Herve, D. Libong, et al., J. Chromatogr. B 807 (2004) 335.

[10] T. Gunnar, K. Ariniemi, P. Lillsunde, J. Mass Spectrom. 41 (2006) 741.

[11] M. Cheze, G. Duffort, M. Deveaux, G. Pepin, Forensic Sci. Int. 153 (2005) 3.

[12] H. Miyaguchi, K. Kuwayama, K. Tsujikawa, T. Kanamori, et al., Forensic Sci. Int. 157 (2006) 57.

[13] M.A. ElSohly, W. Gul, T.P. Murphy, B. Avula, I.A. Khan, J. Anal. Toxicol. 31 (2007) 505.

[14] G. Ngwa, D. Fritch, K. Blum, G. Newland, J. Anal. Toxicol. 31 (2007) 369.

[15] S. McClean, E.J. O'Kane, W.F. Smyth, Electrophoresis 21 (2000) 1381.

[16] G. Vanhoenacker, F. de l'Escaille, D. de Keukeleire, P. Sandra, J. Pharm. Biomed. Anal. 34 (2004) 595.

[17] X. Zhu, M.W. Kamande, S. Thiam, C.P. Kapnissi, et al., Electrophoresis 25 (2004) 562.

[18] J. Hou, S.A.A. Rizvi, J. Zheng, S.A. Shamsi, Electrophoresis 27 (2006) 1263.

[19] M. Kleinschnitz, M. Herderich, P. Schreier, J. Chromatogr. B 676 (1996) 61.

[20] A. Bugey, S. Rudaz, C. Staub, J. Chromatogr. B 832 (2006) 249.

[21] M. Kagan, M. Chlenov, C.M. Kraml, J. Chromatogr. A 1033 (2004) 321.

[22] K. Sato, Y. Mizuno, K. Kobayashi, T. Sano, et al., Jpn. J. Forensic Toxicol. 18 (2000) 32.

[23] P. Hatsis, S. Brombacher, J. Corr, P. Kovarik, D.A. Volmer, Rapid Commun. Mass Spectrom. 17 (2003) 2303.

[24] U. Khasanov, U. Rasulev, D. Usmanov, S. Iskhakova, U. Nabiev, J. Pharm. Biomed. Anal. 37 (2005) 1125.

[25] Y. Hsieh, K. Merkle, G. Wang, J. M. Brisson, A. Korfmacher, Anal. Chem. 75 (2003) 3122.

[26] I. Marchi, S. Rudaz, M. Selman, J.L. Veuthey, J. Chromatogr. B 845 (2007) 244.

[27] M. Takino, S. Daishima, T. Nakahara, Rapid Commun. Mass Spectrom. 17 (2002) 383.

[28] M. Takino, S. Daishima, N. Taketoshi, J. Chromatogr. A 1001 (2003) 67.

[29] M. Takino, S. Daishima, T. Nakahara, Rapid Commun. Mass Spectrom. 17 (2003) 1965.

[30] C. Yang, J. Henion, J. Chromatogr. A 970 (2002) 155.

[31] H.B. Theron, M.J. van der Merwe, K.J. Swart, J.H. van der Westhuizen, Rapid Commun. Mass Spectrom. 21 (2007) 1680.

[32] M.J. Greig, B. Bolanos, T. Quenzer, M.R. Bylund Jessica, Rapid Commun. Mass Spectrom. 17 (2003) 2763.
[33] C.L. Winek, W.W. Wahba, C.L. Winek, T.W. Balzer, Forensic Sci. Int. 122 (2001) 107.
[34] M. Schulz, A. Schmoldt, Pharmazie 58 (2003) 447.
[35] A. Miki, M. Tatsuno, M. Katagi, M. Nishikawa, H. Tsuchihashi, J. Anal. Toxicol. 26 (2002) 87.
[36] M. Laloup, M.D.M. Ramirez Fernandez, G. de Boeck, M. Wood, et al., J. Anal. Toxicol. 29 (2005) 616.
[37] S. Huq, K. Kallury, M. Garriques, LCGC North Am. (2005) 64.
[38] S. Hegstad, E.L. Oeiestad, U. Johansen, A.S. Christophersen, J. Anal. Toxicol. 30 (2006) 31.
[39] M. Walles, W.M. Mullett, J. Pawliszyn, J. Chromatogr. A 1025 (2004) 85.
[40] T. Gunnar, K. Ariniemi, P. Lillsunde, J. Chromatogr. B 818 (2005) 175.
[41] M. Villain, M. Concheiro, V. Cirimele, P. Kintz, J. Chromatogr. B 825 (2005) 72.
[42] O. Quintela, A. Cruz, A. de Castro, M. Concheiro, M. Lopez-Rivadulla, J. Chromatogr. B 825 (2005) 63.
[43] O. Quintela, A. Cruz, M. Conchelro, A. de Castro, M. Lopez-Rivadulla, Rapid Commun. Mass Spectrom. 18 (2004) 2976.
[44] C. Kratzsch, O. Tenberken, F.T. Peters, A.A. Weber, et al., J. Mass Spectrom. 39 (2004) 856.
[45] N.L. Bochkareva, I.N. Glazkov, I.A. Revelsky, J. Anal. Chem. 61 (2006) 1082.
[46] E. Chapuzet, N. Mercier, S. Bervoas-Martin, B. Boulanger, et al., S. T. P. Pharma Pratiques 7 (1997) 169.
[47] P. Hubert, J.J. Nguyen-Huu, B. Boulanger, E. Chapuzet, STP Pharma Pratiques 13 (2003) 101.
[48] P. Hubert, P. Chiap, J. Crommen, B. Boulanger, et al., Anal. Chim. Acta. 391 (1999) 135.
[49] C.E. Huggins, Ann. Surg. 160 (1964) 643.
[50] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019.
[51] R. Bonfiglio, R.C. King, T.V. Olah, K. Merle, Rapid Commun. Mass Spectrom. 13 (1999) 1175.
[52] D.T.T. Nguyen, D. Guillarme, S. Rudaz, J.L. Veuthey, J. Sep. Sci. 31 (2008) 1050.
[53] J.A. Syage, K.A. Hanold, T.C. Lynn, J.A. Horner, R.A. Thakur, J. Chromatogr. A 1050 (2004) 137.
[54] D. Guillarme, D.T.T. Nguyen, S. Rudaz, J.L. Veuthey, Eur. J. Pharm. Biopharm. 66 (2007) 475.
[55] D.B. Robb, M.W. Blades, J. Am. Soc. Mass Spectrom. 16 (2005) 1275.
[56] A. Raffaelli, A. Saba, Mass Spectrom. Rev. 22 (2003) 318.
[57] D.B. Robb, T.R. Covey, A.P. Bruins, Anal. Chem. 72 (2000) 3653.
[58] D.B. Robb, M.W. Blades, J. Am. Soc. Mass Spectrom. 17 (2006) 130.
[59] K.A. Hanold, S.M. Fischer, P.H. Cormia, C.E. Miller, J.A. Syage, Anal. Chem. 76 (2004) 2842.
[60] E. Rozet, A. Ceccato, C. Hubert, E. Ziemons, et al., J. Chromatogr. A 1158 (2007) 111.
[61] C.T. Viswanathan, S. Bansal, B. Booth, A.J. DeStefano, et al., J. Am. Assoc. Pharm. Sci. 24 (2007) E30.
[62] N. Jourdil, J. Bessard, F. Vincent, H. Eysseric, G. Bessard, J. Chromatogr. B 788 (2003) 207.
[63] W.F. Smyth, C. Joyce, V.N. Ramachandran, E. O'Kane, D. Coulter, Anal. Chim. Acta 506 (2004) 203.

[64] K. Yu, D. Little, R. Plumb, B. Smith, Rapid Commun. Mass Spectrom. 20 (2005) 544.
[65] O. Quintela, F.L. Sauvage, F. Charvier, J.M. Gaulier, et al., Clin. Chem. 52 (2006) 1346.
[66] C. Moore, C. Coulter, K. Crompton, M. Zumwalt, J. Anal. Toxicol. 31 (2007) 596.
[67] M.J. Bogusz, R.D. Maier, K.D. Kruger, W. Fruchtnicht, J. Chromatogr. B 713 (1998) 361.
[68] M. Kollroser, C. Schober, J. Pharm. Biomed. Anal. 28 (2002) 1173.

Chapitre 3 : Conclusions et perspectives

Conclusions et perspectives

Lors de ce travail de thèse, divers aspects de l'analyse par LC-MS de composés pharmaceutiques dans des matrices biologiques ont été étudiés.
Le développement de la préparation d'échantillon dans le cadre d'une analyse multi-analytes a été investigué et une méthode a été proposée pour sa simplification. L'utilisation d'un nombre restreint de composés représentatifs de la totalité des substances a permis de réduire le temps ainsi que le nombre d'expériences nécessaires au développement, sans altérer la qualité de la méthode finale. Cette dernière s'est en effet avérée adaptée à l'extraction de la totalité des substances représentées. En revanche, l'utilisation des composés représentatifs ne convient pas à l'évaluation des effets matrice.

Ces derniers ont fait l'objet d'une étude en ESI, APCI et plus particulièrement APPI, celle-ci étant globalement moins encline aux effets matrice que ses deux consœurs. L'APPI a été présentée comme une alternative très intéressante à l'ESI pour l'ionisation de composés pharmaceutiques, puisque pouvant permettre des sensibilités comparables tout en limitant les effets d'altération du signal.

Les effets matrices ont également fait l'objet d'une seconde étude proposant une classification exhaustive lors d'une analyse d'échantillons d'urine et de plasma par SPE-LC-MS. Dix-huit cas ont été distingués et classés dans un organigramme permettant l'identification de l'effet matrice rencontré par simple cheminement sur l'arborescence. D'un point de vue pratique, seules quatre expériences sont requises pour l'attribution des effets matrice *via* cet organigramme, rendant l'identification simple, rapide et peu onéreuse.

Lors de la préparation d'échantillons plasmatiques par SPE couplée en ligne à la LC, une précipitation de protéines préalable à l'injection de l'échantillon dans le système analytique est nécessaire. Ce geste, aussi simple que la dilution largement utilisée, a permis de considérablement allonger la durée de vie de la colonne analytique, de drastiquement diminuer – voire totalement éliminer en APPI – les effets matrice et, finalement, de niveler la qualité de la préparation d'échantillon.

L'utilisation de l'APPI comme alternative à l'ESI pour l'ionisation de benzodiazépines a ensuite été illustrée par la validation d'une méthode SPE-LC-MS pour le dosage de l'alprazolam, du flunitrazepam et de leurs principaux métabolites dans le sang hémolysé. La méthode développée a permis d'atteindre des limites de sensibilité de l'ordre du nanogramme par millilitre avec le MS simple quadripôle à disposition.

Le temps nécessaire au rendu des résultats étant le point clé dans de nombreux domaines (développement du médicament, lutte contre le dopage, TDM, toxicologie, *etc.*), l'UHPLC est devenu

un outil de choix. En revanche, cette technique requérant l'utilisation de supports à faible diamètre de particules, la préparation d'échantillon devient une étape cruciale. De nombreuses applications ont déjà été publiées en UHPLC, mais aucune étude systématique sur l'adéquation de la préparation d'échantillon avec ce type de méthode séparative n'est parue à ce jour. Il serait donc intéressant de comparer les diverses procédures possibles pour la préparation d'échantillon. Par ailleurs, cette dernière devenant également l'étape limitante en terme de temps, il conviendrait d'investiguer les approches de préparation rapide de l'échantillon.

Dans cette optique, des méthodes de préparation d'échantillon automatisées sont disponibles, telles que la LLE sur cartouche (SLE) ainsi que la PP sur cartouche. La SLE semble être une voie intéressante, alliant les avantages de la LLE (rendement d'extraction, propreté de l'échantillon) à une automatisation augmentant considérablement la productivité. La PP automatisée permet quant à elle d'associer l'élimination d'une grande partie du contenu protéique de l'échantillon à une productivité accrue. Il serait également intéressant d'évaluer la combinaison de deux types de préparation d'échantillon (PP + SPE off-line par exemple) pour une plus grande propreté de l'échantillon injecté en vue de son analyse par UHPLC. Précisément, des supports d'extraction permettant d'effectuer une PP préalablement à une extraction SPE sur la même cartouche sont récemment apparus. Ces supports, appelés HybridSPE, ont en outre l'avantage d'éliminer une forte proportion du contenu phospholipidique de la matrice, reconnu pour participer, conjointement aux protéines, aux effets d'altération du signal MS.

A ce titre, la présence d'interférents dans l'échantillon injecté est non seulement un sujet de première importance concernant la séparation mais également en regard des effets matrice engendrés en MS. Ces derniers étant partiellement liés à la partie séparative, il mériterait également d'en étudier la portée dans le cadre d'analyses d'échantillons biologiques en UHPLC-MS.

Finalement, la sélectivité étant un sujet primordial lors du développement de méthode et de la validation, un gain notable peut être apporté par le recours à d'autres types d'analyseurs de masse, tels que le triple quadripôle ou la trappe d'ion, par exemple. Il serait donc intéressant de d'évaluer les tenants et les aboutissants de l'utilisation de ces outils pour l'analyse de composés pharmaceutiques en UHPLC-MS.

Annexes

Article IV

Fast analysis of doping agents by Ultra Performance Liquid Chromatography

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G.I.T. Laboratory Journal, Europe, 11 (2007) 14-15

For several years, sport federations have decided to be very stringent against doping. Therefore, athletes are tested for the presence of forbidden substances mainly in urine, but also increasingly in blood, during competition and training periods (out-of-competition tests). Sample collection and analysis are strictly regulated by the World Anti-Doping Agency (WADA) and are only performed by accredited staff-members and laboratories. Today, it is estimated that there are more than 150'000 tested samples per year over the world, mainly in Europe and this number is doomed to rapidly increase. The constraints on response time are becoming more difficult with results needed within 24 or 48 hours. Generally, the analysis of a sample occurs in two steps : a rapid screening is first operated and in the case of a positive result, a confirmatory analysis, together with quantitative measurements for some threshold substances, is performed. Therefore, particularly at the screening level, powerful, rapid and simple analytical methods, as generic as possible, are absolutely necessary to deal with the increasing number and complexity of controlled samples.

Analytical process

Due to the complexity of blood (i.e. plasma) and urine matrices and the low levels of screened substances among a large amount of endogenous compounds, sample preparation is mandatory in order to remove usual interferents (such as salts, proteins, fatty acids, etc) and to lower the limits of detection. Thus, the analytical process is made up of four distinct steps, namely : sample preparation, separation, detection and data analysis. Today, liquid chromatography coupled with mass spectrometry (LC-MS) is considered as the gold standard for analyzing drug substances at low concentration levels, even if other analytical techniques such as gas chromatography and capillary electrophoresis coupled with different detectors as well as immunoassays remain of great interest. However, analysis time is often a critical factor due to complicated and tedious sample preparation steps and LC separations. These must be reduced in order to increase the throughput of these assays.
The main objective of this paper is to describe a complete strategy for the rapid and selective analysis of doping substances in biological matrices.

Sample preparation

With urine matrices, different sample preparation procedures can be used according to both screened substances and selected analytical method. However, due to the low amount of investigated analytes in biological samples, the "dilute and shoot" procedure [1], as well as the direct injection of filtered urine is generally not recommended even with a powerful LC-MS/MS technique. Moreover, automated sample preparation in LC-MS (e.g. column switching), which been successful in drug analysis [2], can not be considered as a valuable strategy in doping control to avoid any carry over risk and to ensure sample independence. Besides, an on-line solid phase extraction (SPE) procedure

is adapted to a dedicated analysis (e.g. therapeutic drug monitoring, pharmacokinetic study) but not to the analysis of a broad range of analytes present at very low concentrations. Furthermore, the same sample could require the use of different and orthogonal analytical methods to unambiguously prove the presence of a prohibited substance. Therefore, off-line sample preparation procedures, such as liquid-liquid extraction (LLE) and off-line SPE, are preferred. The former has been largely used and presents advantages in terms of efficiency, simplicity and cost. However, it suffers of some drawbacks such as the long time required for performing a complete procedure and a lack of possibilities for automation. For these reasons, SPE is considered as the method of choice.

With plasma samples, the same issues arise and, therefore, a simple protein precipitation (PP) prior to analysis is often not sufficient. Different specific sorbents such as affinity columns and proteome partitioning kits have recently been developed for the extraction of particular compounds such as doping substances. As reported in the World Anti-Doping Agency (WADA) list available on the internet [3], these compounds are of different nature and comprise various families such as stimulants, anabolic steroids, diuretics, β-blockers, opiates, cannabinoids, glucocorticosteroids, □2-agonists, proteins, anti-estrogens, hormones, etc. Thus, many of the latter possess hydrophobic moieties as well as basic or acidic functions which can be extracted with hydrophobic or mixed mode materials for gaining selectivity [4]. Furthermore, new extraction sorbents used in SPE are now compatible with the direct injection of blood samples, since proteins are excluded when large loading flow rates are applied [5].

In conclusion, off-line SPE is a generic approach which can be used for urine and blood samples. It is versatile, easy to perform and can be compatible with several analytical methods. Furthermore, its automation has already been described [6] and a large number of samples can be prepared using 96- or 384-wells plates drastically reducing the average sample preparation time (e.g. 96 samples extracted in less than three hours, all steps included).

LC-MS analysis

As mentioned above, LC-MS is presently considered as the gold standard for such analyses. For performing rapid and efficient separations, different strategies have been implemented in the last five years [7]. One attractive alternative is the use of sub-2µm particles packed in short chromatographic columns. With this material, it is possible to significantly reduce the analysis time, while maintaining efficiency and sensitivity constant [8]. For example, using the appropriate fundamental equations of chromatography [9], the analysis time can be reduced by a factor 10 to 20 maintaining a good resolution [10] in comparison with conventional methods (Figure 1) for the analysis of a set of doping substances.

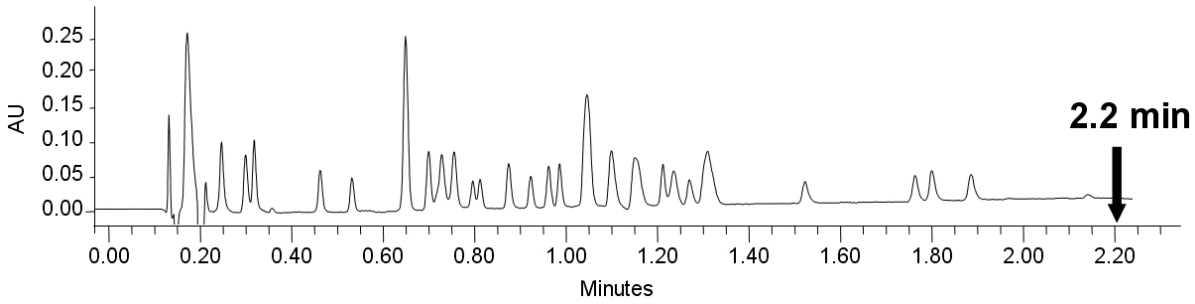

Figure 1 :UPLC-UV separation of 36 substances comprising stimulants, diuretics, and β-blockers.

It can be noted that due to the thinness of peaks, detectors (i.e. UV-Vis and MS) with high acquisition rates (> 40Hz) are mandatory. However, these small particles induce a large back pressure and a dedicated instrumentation compatible with very high pressures (i.e. 1000 bar) is required. Since 2004, instruments which allow performing separations of complex mixtures within a couple of minutes (Figure 2) were commercialized (e.g. Ultra Performance Liquid Chromatography (UPLC)).

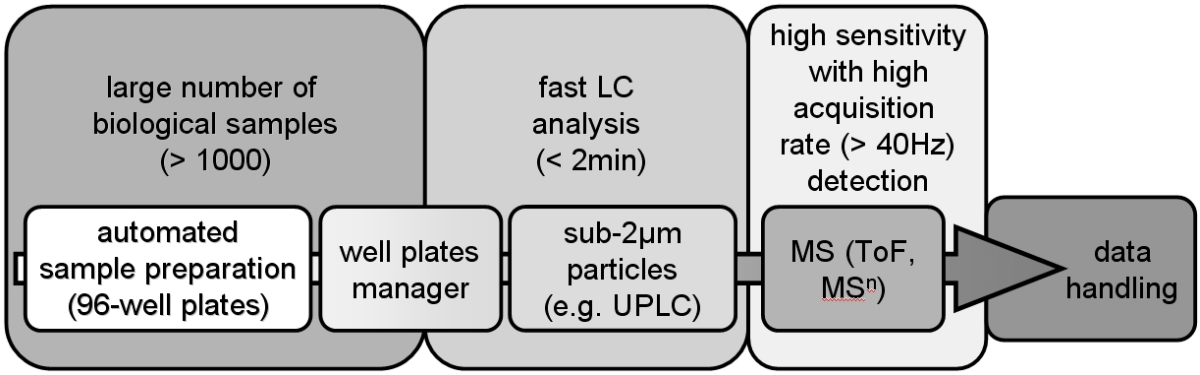

Figure 2 : Schematic representation of proposed solution for fast analysis.

Conclusion

For screening and confirmatory analysis of doping substances in biological matrices (i.e. urine and plasma), a generic approach can be easily implemented to reduce the time response delivery while maintaining high chromatographic performance. For this purpose, an off-line SPE performed automatically in 96- or 384-well plate formats can be directly coupled to a UPLC-MS system as shown in Figure 2. With this strategy, it is possible to analyze 100 samples per day (8 hours) per instrument corresponding to a result each 5 minutes. Of course, this procedure could be implemented in systems which permit the injection of prepared well-plates and be compatible with using columns

packed with sub-2µm particles (in terms of maximal reachable pressure and detection acquisition rates).

Acknowledgements

The authors gratefully acknowledge Waters for the loan of the UPLC system and the generous gift of Acquity columns.

References

1. Peng L., McGinley M., LCGC North Am., 21 (2006).
2. Souverain S., Rudaz S., Veuthey J.-L., J. Chromatogr. B. 801, 141-56 (2004).
3. WORLD ANTI-DOPING AGENCY. Prohibited list. In : WADA website [on-line]. http://www.wada-ama.org/en/dynamic.ch2?pageCategory.id=370 (accessed on December 14th, 2006).
4. Jenkins K.M., Young M.S., Mallet C.R., Elian A.A., J. Anal. Toxicol. 28, 50-58 (2004).
5. Souverain S., Rudaz S., Veuthey J.-L., G.I.T. Lab. J. 02, 80-82 (2003).
6. D.A.Wells, High Throughput Bioanalytical Sample Preparation : Methods and Automation Strategies, Elsevier, New York, 2003.
7. Rudaz S., Geiser L., Guillarme D., Veuthey J.L., Chimia 59, 303-07 (2005).
8. Nguyen D.T.T., Guillarme D., Rudaz S., Veuthey J.L., J. Chromatogr. A. 1128, 105-13 (2006).
9. LABORATORY OF ANALYTICAL PHARMACEUTICAL CHEMISTRY. HPLC calculator download. In : LCAP website [on-line]. Site updated on November 13th, 2006. http://www.unige.ch/sciences/pharm/fanal/divers/downloads.php (accessed on December 14th, 2006).
10. Nguyen D.T.T., Guillarme D., Rudaz S., Veuthey J.L., J. Sep. Sci. 29, 1836-48 (2006).

Abstract I

Evaluation of matrix effects after on-line extraction procedures with commercially available ESI, APCI and APPI sources

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ABSTRACT

Over the past decade, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) sources became the classical interfaces used for liquid chromatography hyphenated to mass spectrometry (LC-MS) for performing fast, selective and sensitive analysis of pharmaceuticals in biological fluids. Atmospheric pressure photoionization (APPI) is the latest source among API techniques which allows to further extend the field of application of LC-MS to low polarity molecules.

When LC-MS analyses are performed on complex biological matrices, an alteration of the analytical response can occur. This phenomenon called 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. These matrix effects have already been widely reported in the literature especially for ESI and APCI but to the best of our knowledge, only a few studies have been published with APPI.

In order to overcome matrix effects, sample clean-up procedures must be operated to remove potential interferents. They are divided in two main groups namely off-line and on-line sample preparations. The latter performs the sample extraction directly in the LC system by the mean of dedicated supports. Three kinds of on-line extraction supports such as restricted access material (RAM), large particle support (LPS) and monoliths, respectively, allow direct injection of biological samples. Furthermore, to increase extraction support and column lifetimes, a sample pre-treatment could be assessed prior to the injection (i.e. protein precipitation).

In this study, matrix effect was evaluated with a post-column infusion system with commercially available ESI, APCI, and APPI sources coupled to a single quadrupole mass spectrometer. Four commercially available extraction supports (one RAM, two LPS and one monolith) for on-line sample preparation of human plasma were used in the column-switching configuration. Direct injection and sample pre-treatment (protein precipitation with acetonitrile) were performed before the injection in the LC-MS system. Each extraction support was investigated with three pharmaceutical compounds and their primary metabolites : methadone (MTD), 2-ethyldiene-1,5dimethyl-1,3-diphenylpyrrolidine (EDDP), fluoxetine (FLX), norfluoxetine (NFLX), flunitrazepam (FLZ) and norflunitrazepam (NFLZ), one vitamin, vitamin D3 (VD3) and one pesticide, metalaxyl (MTX).

Results were analyzed with the help of principal component analysis (PCA) and demonstrated that protein precipitation reduced drastically matrix effects in all cases. APPI exhibited in most of the cases lower sensitivity to endogenous compounds than APCI and ESI. Considering most analytes used, ESI is the most sensitive source but shows also the most sensitivity toward matrix effects.

Abstract II

Chemometric tools to simplify method development : screening of doping agents in urinary samples

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ABSTRACT

Analyses in anti-doping control occur in two steps. First, a generic fast screening analysis is used to determine the presence of a large number of forbidden compounds in urine. Second, in the case of positive result, a specific procedure (confirmatory analysis) has to be performed to quantify the substance(s).

Screening method development is tedious and time consuming due to the necessity to optimize the sample preparation of a large quantity of compounds. The use of chemometric tools is therefore proposed to reduce the number of tested analytes in method development by choosing representative compounds of the whole set.

In this study, a group of thirty-six doping agents consisting of diuretics and beta-blockers was used. A standard solution containing all analytes was loaded, washed and eluted with four different SPE sorbents. All fractions were collected and each compound was retrieved by LC-ESI-MS. In order to bring out differences among compounds, a multivariate analysis approach was used. A small number of groups emerged and subsequent method development was performed by selecting representative compounds in the obtained clusterisation.

Abstract I

Traitement et analyse d'échantillons plasmatiques en chromatographie liquide / spectrométrie de masse : étude des effets de suppression de signal

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2ème PRIX DU JURY

ABSTRACT

Les effets de suppression de signal en spectrométrie de masse lors de l'analyse d'échantillons biologiques peuvent être dus à la présence de matériel endogène divers tel que protéines, lipides, sels, etc. Afin de limiter ces effets de suppression de signal sur la réponse analytique mesurée, une étape de traitement d'échantillon est nécessaire. Dans le cas de la chromatographie en phase liquide couplée à la spectrométrie de masse (LC-MS), cette étape peut être effectuée en mode différé, avant l'injection de l'échantillon dans le système analytique (off-line) ou en mode couplage par extraction en phase solide (SPE on-line).

Ce travail porte sur l'évaluation de la qualité de diverses préparations d'échantillons (SPE on-line) préalables à une analyse LC-MS [1,2]. Deux sources d'ionisation à pression atmosphérique, couramment utilisées en analyse pharmaceutique, l'electrospray (ESI) et l'ionisation chimique à pression atmosphérique (APCI), ont été évaluées. La photo-ionisation à pression atmosphérique (APPI), permettant d'élargir le domaine d'application de la LC-MS aux molécules peu voire non-polaires, a également été étudiée.

[1] Souverain S., Rudaz, S., Veuthey J.-L. *J. Chrom. A*, 1058 (2004) 61-66

[2] Souverain S., Rudaz, S., Veuthey J.-L. *J. Pharm. Biomed. Anal.*, 35 (2004) 913-920

Abstract II

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 effect on mass spectrometry (MS) response was investigated with atmospheric pressure ionization (API) sources after on-line solid-phase extraction (SPE) of human plasma. A post-column infusion system was used to measure the MS signal alterations of eight analytes chosen as model compounds. On-line SPE was evaluated with one restricted access material (RAM), two large particle supports (LPS) and one monolith. A sample pre-treatment (protein precipitation (PP)) and a direct injection (dilution) were tested. Principal component analysis (PCA) was performed to simplify data presentation and interpretation. For all extraction materials and ionization sources, PP was found mandatory for significantly reducing signal modification due to endogenous components eluted from the extraction support. Regarding sensitivity towards matrix effects after PP, APPI was globally the least sensitive ionization mode while ESI was the most sensitive.