Sensitivity improvements in capillary electrophoresis-mass spectrometry for clinical and forensic toxicology

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Sensitivity Improvements in Capillary Electrophoresis – Mass Spectrometry for Clinical and Forensic Toxicology

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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".

Qui triomphe de lui-même possède la force

Lao Tseu

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Preface

This thesis work was carried out in the School of Pharmaceutical Sciences at the University of Geneva, and was achieved in the group of pharmaceutical analytical chemistry under the co-direction of Prof. Jean-Luc Veuthey and Prof. Serge Rudaz, and the supervision of Julie Schappler, Ph.D.

The very first attempt in hyphenating capillary electrophoresis (CE) with mass spectrometry (MS) within the group was performed by Emmanuel Varesio, Ph.D., who presented the application of CE-MS to the analysis of amphetamines ("Analyse des dérivés de l'amphétamine par électrophorèse capillaire", Thesis No. 3053, University of Geneva, 1999). This work was followed by the thesis of Laurent Geiser, Ph.D., who further investigated the potential of CE-MS for the analysis of pharmaceutical compounds ("Développement et validation de méthodes analytiques pour l'analyse de composés pharmaceutiques par électrophorèse capillaire couplée à un spectrophotomètre UV ou à un spectromètre de masse », Thesis No. 3442, University of Geneva, 2003). Both works studied the hyphenation of CE with MS *via* electrospray ionization (ESI) source equipped with a sheath-flow interface. More recently, Grégoire Bonvin has been evaluating the technical developments in CE-ESI-MS hyphenation, focusing on the performance of the sheathless interface (ongoing thesis).

The application of CE-MS to clinical and forensic toxicology was first investigated by Andrea Baldacci, Ph.D., with multi-stage ion trap MS for the elucidation of the metabolism of drugs ("Capillary electrophoresis – electrospray ionization multi-stage mass spectrometry for the identification of phase I and phase II metabolites of drugs in biological samples", Thesis No. 3732, University of Geneva, 2006, in collaboration with the Department of Clinical Pharmacology, University of Bern). The work of Julie Schappler, Ph.D., highlighted the potential of CE-MS for the quantitation of drugs of abuse in biosamples with validated procedures ("Analyse de composés pharmaceutiques par électrophorèse capillaire couplée à des techniques de détection alternatives", Thesis No. 3937, University of Geneva, 2008). Finally, Aline Staub Spörri, Ph.D., combined CE with a time-of-flight mass analyzer for the analysis of toxicologically relevant proteins; *inter alia*, human growth hormone and hemoglobin-based oxygen carriers ("Analyse de protéines intactes par électrophorèse capillaire couplée à un spectromètre de masse à temps de vol", Thesis No. 4288, University of Geneva, 2011).

The analysis in body fluids raised the importance of two relevant topics, *i.e.*, the sample preparation and the evaluation of matrix effects. In parallel to these works in CE-MS, the two items were also deeply studied by Sandrine Fleury-Souverain, Ph.D. ("Extraction en ligne sur support solide pour l'analyse de composés pharmaceutiques contenus dans des matrices biologiques par chromatographie liquid-spectrométrie de masse", Thesis No. 3520, University of Geneva, 2004), and Ivano Marchi, Ph.D. ("Influence de la préparation d'échantillons biologiques en LC-API-MS", Thesis No. 4047, University of Geneva, 2008), who both used liquid chromatography-MS. These topics were also studied in the thesis of Flavia Badoud, Ph.D, with the evaluation of ultra-high pressure liquid chromatography and MS for screening and quantitation purposes in anti-doping analysis ("Application de la chromatographie liquide à ultra-haute pression couplée à un spectromètre de masse quadripôle à temps de vol pour l'analyse antidopage", Thesis No. 4376, University of Geneva, in collaboration with the Swiss Anti-Doping Laboratory, University of Lausanne, 2011).

The present thesis represents a straight continuation of these previous works which highlighted the potential of CE-MS in bioanalysis. Over the last decade, new challenges have been emerging in

clinical and forensic toxicology, leading to innovative developments of powerful analytical strategies. Meantime, a growing concern regarding the protection of both environment and human's health also emerged with the rise in power of environmentally sustainable chemistry. After a 20-year history, CE-MS has been receiving an increased attention with numerous attempts in performance improvement; however, its use is still rather limited in clinical and forensic toxicology. Due to the very low volumes injected, CE-MS suffers from a lower analytical sensitivity than chromatography coupled to MS, leading to inadequate limits of detections. Furthermore, this low analytical sensitivity is also crucial in screening approaches since an unacceptable number of false-negative results are encountered due to a lower diagnostic sensitivity of CE-MS-based assays. This work thus focused on the development of highly sensitive CE-MS procedures to integrate this technique as a competitive analytical tool in clinical and forensic toxicology.

The present manuscript is composed of six sections. The first section (**Chapter I**) provides the general introduction on clinical and forensic toxicology, including history and definitions, bioanalytical procedures, and strategies used in daily practice. Important notions in biosamples selection and collection, sample pre-treatment, state-of-the-art analytical techniques, and data treatment and interpretation for systematic toxicological analysis and quantitation are discussed. Background theory of CE and CE-MS coupling is presented, and its contribution in clinical and forensic toxicology discussed.

The **Chapter II** emphasizes the emergence of novel miniaturized sample preparation techniques. Although widely used in toxicology, extraction techniques such as liquid-liquid extraction and solid-phase extraction suffer from significant drawbacks which promoted the development of miniaturized procedures, allowing for a decrease of the solvent and/or sample consumption. This section focuses on the microextraction techniques, in which the amount of organic solvent is significantly reduced. The principle of liquid-based and solid-based microextractions is presented and the benefits of their combination with CE analysis discussed (**Article I**). One of the most promising liquid-based microextractions, the dispersive liquid-liquid microextraction, was evaluated in the present work and is exposed (**Article II**). A recent solid-based microextraction, the disposable pipette extraction, was also investigated and the results are summarized in this chapter.

The third section (**Chapter III**) is devoted to the implementation of a two-step CE-MS strategy for multi-target screening and quantitation of drugs of abuse in urine. The analytical prerequisites to competitively integrate a CE-MS workflow in clinical and forensic toxicology are first discussed. The proposed improvements for sensitivity, selectivity, and throughput enhancement are then presented, with the implementation of an on-line sample preconcentration technique, the use of coated capillaries, the modification of the CE-ESI-MS interface geometry, as well as the use of high sensitive mass analyzers (**Article III**).

Leaving the world of low-molecular weight xenobiotics, the **Chapter IV** presents the contribution of CE-MS for intact protein analysis in forensic toxicology with the determination of carbohydrate-deficient transferrin (CDT), an indirect biomarker of chronic alcohol consumption. The analysis of intact glycoproteins by CE-MS involves different strategies and issues that are exposed and discussed. This chapter introduces the development of a CE-MS-friendly method for the determination of CDT to improve both sensitivity and selectivity of the CE-UV method currently used in routine analysis (**Article IV**).

The **Chapter V** presents the final conclusions that were raised during the present thesis and the covered topics. Future outlooks are depicted and proposed in view of a daily use of CE-MS in clinical and forensic toxicology.

Finally, the last section (**Chapter VI**) provides the Appendices concerning additional works to the present thesis.

List of abbreviations

Abbreviations used through the manuscript are listed in the present section and classified according to the chapter where they appear. Common abbreviations encountered in the whole manuscript are presented separately.

Common abbreviations

Ab	Antibody
AEME	Anhydroecgonine methyl ester
Ag	Antigen
BGE	Background electrolyte
BZD	Benzodiazepine
CE	Capillary electrophoresis
CEC	Capillary electrochromatography
CGE	Capillary gel electrophoresis
CIEF	Capillary isoelectric focusing
COC	Cocaine
CZE	Capillary zone electrophoresis
DBS	Dried blood spot
D-PX	D-propoxyphene
DS	Dextran sulfate
EDDP	2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolydine
EK	Electrokinetic
EMA	European Medicines Agency
EOF	Electro-osmotic flow
ESI	Electrospray ionization
EY	Extraction yield
FDA	Food and Drug Administration
FWHM	Full width at half maximum
GC	Gas chromatography
GHB	γ-hydroxybutyric acid
HD	Hydrodynamic
HILIC	Hydrophilic interaction liquid chromatography
HRMS	High resolution mass spectrometry
ICH	International Conference on Harmonization
i.d.	Internal diameter
Ig	Immunoglobulin
IS	Internal standard
LC	Liquid chromatography
LLE	Liquid-liquid extraction
LLOQ	Lower limit of quantitation
LOD	Limit of detection
MALDI	Matrix-assisted laser desorption/ionization
6-MAM	6-monoacetylmorphine
MBDB	N-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine
MDA	3,4-methylenedioxyamphetamine
MDEA	3,4-methylenedioxyethylamphetamine
MDMA	3,4-methylenedioxymethamphetamine
MEEKC	Microemulsion electrokinetic chromatography
MEKC	Micellar electrokinetic chromatography
MS	Mass spectrometry
MTD	Methadone
OLS	Ordinary least square
PB	Polybrene
PE	Process efficiency
PP	Protein precipitation
PVS	Poly(vinylsulfonate)
QqQ	Triple quadrupole
QTOF	Quadrupole time-of-flight
RAM	Restricted access materials
RE	Recovery of extraction
RPLC	Reversed-phase liquid chromatography
RSD	Relative standard deviation
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SFSTP	Société Française des Sciences et Techniques Pharmaceutiques
SIM	Selected ion monitoring
SPE	Solid-phase extraction
SPME	Solid-phase microextraction
SRM	Selected reaction monitoring
STA	Systematic toxicological analysis
TFA	Trifluoroacetic acid
THC	Tetrahydrocannabinol
TIC	Total ion current
TOF	Time-of-flight
UHPLC	Ultra-high pressure liquid chromatography
UV/Vis	Ultraviolet/Visible

Chapter I.

AACB	Australasian Association of Clinical Biochemists
AACT	American Academy of Clinical Toxicology
AAFS	American Academy of Forensic Sciences
ABP	Athlete Biological Passport
ACB	Association of Clinical Biochemists
AGREE	Appraisal of Guidelines for Research and Evaluation
AMDIS	Automated Mass Spectral Deconvolution and Identification System
APCI	Atmospheric pressure chemical ionization
API	Atmospheric pressure ionization
APPI	Atmospheric pressure photo-ionization
AS/NZS	Australian/New Zealand Standard
ASCEPT	Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists
CAOD	Committee on Alcohol and Other Drugs
CI	Chemical ionization
CID	Collision-induced dissociation
CLSI	Clinical and Laboratory Standards Institute
CNBH	Collège National de Biochimie des Hôpitaux
CPDD	College on Problems of Drug Dependence
DAD	Diode array detector
DDA	Data-dependent acquisition
DFSA	Drug-facilitated sexual assault
DGKC	Deutsche Gesellschaft für Klinische Chemie
DGLM	Deutsche Gesellschaft für Laboratoriumsmedizin
DOB	2,5-dimethoxy-4-bromoamphetamine
DUID	Driving under the influence of drugs
DUS	Dried urine spot
ED	Effective dose
EDTA	Ethylenediaminetetraacetic acid
EI	Electron ionization
ELISA	Enzyme-linked immunosorbent assay
EMIT	Enzyme multiplied immunoassay technique
EPR	Enhanced product ion
EtG	Ethyl glucuronide
EWDTS	European Workplace Drug Testing Society
FFLM	Faculty of Forensic and Legal Medicine
FID	Flame ionization detector
FPIA	Fluorescence polarization immunoassay
FT-ICR	Fourier transform ion cyclotron resonance
GTFCh	Gesellschaft für Toxikologische und Forensische Chemie
HBG	Hemoglobin
HCT	Hematocrit
HMOR	Hydromorphone
HPLC	High pressure liquid chromatography
HPTLC	High performance thin-layer chromatography
ILAC	International Laboratory Accreditation Cooperation
IMMS	Ion mobility mass spectrometry
IMS	Ion mobility spectrometry
IOC	International Olympic Committee
ISO	International Organization for Standardization
IT	Ion trap
IUPAC	International Union of Pure and Applied Chemistry
KIMS	Kinetic interaction of microparticles in solution
LD	Lethal dose
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
mCPP	1-(3-chlorophenyl)-piperazine
MCV	Mean corpuscular volume
MRPL	Minimum Required Performance Levels
NACB	National Academy of Clinical Biochemistry
NACE	Non-aqueous capillary electrophoresis
NAPQI	*N*-acetyl-p-benzoquinone imine
NICI	Negative ion chemical ionization
NPD	Nitrogen-phosphorous detector
NPIS	National Poisons Information Service
NSC	National Safety Council
PR	Product ion
POCT	Point-of-care test
RACP	Royal Australasian College of Physicians
RCB	Red blood cells count
RCPA	Royal College of Pathologists of Australasia
RET	Reticulocyte
RIA	Radioimmunoassay
RT	Retention time
SAMHSA	Substance Abuse and Mental Health Services Administration
SCDAT	Swiss Guidelines Committee for Drugs of Abuse Testing
SFBC	Société Française de Biologie Clinique
SFC	Supercritical fluid chromatography
SFMU	Société Française de Médecine d'Urgence
SFTA	Société Française de Toxicologie Analytique
SOFT	Society of Forensic Toxicologists
SOHT	Society of Hair Testing
SRLF	Société de Réanimation de Langue Française
STC	Société de Toxicologie Clinique
SWGTOX	Scientific Working Group for Forensic Toxicology
TD	Toxic dose
TDM	Therapeutic drug monitoring
TIAFT	The International Association of Forensic Toxicologists
TLC	Thin-layer chromatography

HTLC	High temperature liquid chromatography
IAAF	International Association of Athletics Federations
ICP	Inductively coupled plasma
IEC	International Electrotechnical Commission
UHPSFC	Ultra-high performance supercritical fluid chromatography
UKIAFT	United Kingdom and Ireland Association of Forensic Toxicologists
WADA	World Anti-Doping Agency
WDT	Workplace drug testing

Chapter II.

CM	Carrier-mediated
CME	Centrifuge microextraction
DI	Direct immersion
DLLME	Dispersive liquid-liquid microextraction
DMD	Droplet membrane droplet
DPX	Disposable pipette extraction
DSDME	Directly suspended droplet microextraction
dSPE	Dispersive solid-phase extraction
EME	Electro membrane extraction
GAC	Green analytical chemistry
HF-LPME	Hollow-fiber based liquid-phase microextraction
HS	Headspace
IL	Ionic liquid
ILBE	In-line back-extraction
LLLME	Liquid liquid liquid microextraction
LPME	Liquid-phase microextraction
ME	Microextraction
MEPS	Microextraction by packed sorbent
MIP	Molecularly imprinted polymer
OLBE	On-line back-extraction
PT-LLLME	Phase-transfer based liquid liquid liquid microextraction
SBSE	Stir-bar sorptive extraction
SDME	Single drop microextraction
SLM	Supported liquid membrane
USAEME	Ultrasound-assisted emulsification microextraction

Chapter III.

ACCEL	*Accelerator*
ADC	Analog-to-digital converter
AJS	Agilent Jet Stream
FASI	Field-amplified sample injection
FASS	Field-amplified sample stacking
FESI	Field-enhanced sample injection
INIT	*Initiator*
KRF	Kohlrausch regulating function
LE	Leading electrolyte
LVSS	Large-volume sample stacking
oa	Orthogonal acceleration
TE	Terminating electrolyte
tITP	Transient isotachophoresis

Chapter IV.

ALAT	Alanine aminotransferase
ASAT	Aspartate aminotransferase
CDT	Carbohydrate-deficient transferrin
ER	Endoplasmic reticulum
GGT	γ-glutamyltransferase
IEC	Ion-exchange chromatography
IEF	Isoelectric focusing
PEI	Trimethoxysilylpropyl(polyethyleneimine)
p*I*	Isoelectric point
PTM	Post-translational modification
SEC	Size-exclusion chromatography
SMIL	Successive multiple ionic-polymer layer
Tf	Transferrin
WG-CDT	Working group on Standardization of CDT

Publications and scientific communications

The present thesis has been subject to publications in peer-reviewed scientific journals, as well as other formats such as non-peer-reviewed articles, and technical and application notes. The research projects were also presented in national and international conferences in the form of oral or poster communications. Some side projects carried out prior to or during the thesis which led to peer-reviewed publications are also listed hereafter.

Peer-reviewed publications

I. Microextraction techniques combined with capillary electrophoresis in bioanalysis
I. Kohler, J. Schappler, S. Rudaz, Anal Bioanal Chem 405 (2013) 152

II. Dispersive liquid-liquid microextraction combined with capillary electrophoresis and time-of-flight mass spectrometry for urine analysis
I. Kohler, J. Schappler, T. Sierro, S. Rudaz, J Pharm Biomed Anal 73 (2013) 82

III. Highly sensitive capillary electrophoresis-mass spectrometry for rapid screening and accurate quantitation of drugs of abuse in urine
I. Kohler, J. Schappler, S. Rudaz, Anal Chim Acta 780 (2013) 101

IV. New insight in carbohydrate-deficient transferrin analysis with capillary electrophoresis - mass spectrometry
I. Kohler, M. Augsburger, J. Schappler, S. Rudaz, Forensic Sci Int (2013), submitted.

Other publications

I. Single-run separation of closely related cationic and anionic compounds by CE-ESI-MS: application to the simultaneous analysis of melamine and its analogs in milk
I. Kohler, E. Cognard, I. Marchi, D. Ortelli, P. Edder, J.L. Veuthey, S. Rudaz, J. Schappler, Chimia 65 (2011) 389

II. Sample preparation of urine samples prior to CE-MS in toxicological analysis
M. Rovini, J. Schappler, I. Kohler, M. Anzini, J.L. Veuthey, S. Rudaz, J Chem Chem Eng 5 (2011) 583

III. Compatibility of Agilent Jet Stream thermal gradient focusing technology with CE/MS
I. Kohler, J. Schappler, S. Rudaz, H.P. Zimmermann, C. Wenz, Agilent Technologies Technical Note, publication number 5990-9716EN (2012)

IV. Highly sensitive CE-ESI-MS/MS for accurate quantitation of drugs of abuse in bioanalysis using the Agilent 6490 Triple Quadrupole LC/MS System
I. Kohler, J. Schappler, S. Rudaz, M. Greiner, Agilent Technologies Application Note, publication number 5991-2395EN (2013)

V. Microextraction liquide-liquide dispersive combinée à l'électrophorèse capillaire et la spectrométrie de masse pour l'analyse de l'urine
I. Kohler, J. Schappler, T. Sierro, S. Rudaz, CJ'MAG 4 (2012) 3

Side projects

I. Importance of instrumentation for fast liquid chromatography in pharmaceutical analysis
S. Fekete, I. Kohler, S. Rudaz, D. Guillarme, J Pharm Biomed Anal (2013) doi: 10.1016/j.jpba.2013.03.012

II. Advances in hydrophilic interaction liquid chromatography for pharmaceutical analysis
A. Periat, I. Kohler, J.L. Veuthey, D. Guillarme, LCGC Eur 26 (2013) 17

III. Isolation and quantification by high-performance liquid chromatography-ion-trap mass spectrometry of androgen sulfoconjugates in human urine
E. Strahm, I. Kohler, S. Rudaz, S. Martel, P.A. Carrupt, J.L. Veuthey, M. Saugy, C. Saudan, J Chromatogr A 1196-1197 (2008) 153

Oral communications

- Dispersive liquid-liquid microextraction combined with capillary electrophoresis and mass spectrometry for urine analysis
I. Kohler, J. Schappler, T. Sierro, J.-L. Veuthey, S. Rudaz, 26th seminary in Pharmaceutical Sciences, Analytics on the Top, September 2011, Zermatt (Switzerland).

- Microextraction dispersive liquide-liquide combinée à l'électrophorèse capillaire et la spectrométrie de masse pour l'analyse de l'urine
I. Kohler, J. Schappler, T. Sierro, J.-L. Veuthey, S. Rudaz, 15èmes Journées Scientifiques du cCCTA, September 2011, Les Diablerets (Switzerland).

- Dispersive liquid-liquid microextraction combined with capillary electrophoresis and mass spectrometry for urine analysis
I. Kohler, J. Schappler, T. Sierro, J.-L. Veuthey, S. Rudaz, Recent Developments in Pharmaceutical Analysis (RDPA) 2011, September 2011, Pavia (Italy).

- Microextraction dispersive liquide-liquide combinée à l'électrophorèse capillaire et la spectrométrie de masse pour l'analyse de l'urine
I. Kohler, J. Schappler, T. Sierro, J.-L. Veuthey, S. Rudaz, 3ème Journée du Club Jeunes, November 2011, Lyon (France). **Best Scientific Communication Award**.

- Combination of liquid-based microextraction technique with capillary electrophoresis and mass spectrometry for toxicological analysis
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- Highly sensitive capillary electrophoresis-mass spectrometry for rapid screening and accurate quantitation of drugs of abuse in urine
  I. Kohler, J. Schappler, M. Greiner, S. Rudaz, 51st Annual Meeting of the International Association of Forensic Toxicologists (TIAFT) 2013, September 2013, Funchal – Madeira (Portugal).

Résumé de la thèse

L'électrophorèse capillaire (*capillary electrophoresis*, CE), qui met en œuvre la séparation de composés chargés selon leur rapport charge sur taille, est une technique analytique puissante qui présente de nombreux avantages tels qu'une grande efficacité et une faible consommation de solvants et d'échantillon. Cependant, la détection UV/visible généralement utilisée souffre d'un manque de sensibilité en raison de la faible longueur du chemin optique représentée par le diamètre interne du capillaire. Dans le but d'augmenter non seulement la sensibilité mais également la sélectivité de l'analyse, la CE peut être couplée avec la spectrométrie de masse (*mass spectrometry*, MS). La technique d'ionisation par électrospray (*electrospray ionization*, ESI) est la plus utilisée pour réaliser ce couplage, et ce à l'aide d'une interface commerciale basée sur l'utilisation d'un triple-tube permettant l'ajout d'un liquide additionnel. Le couplage CE-MS, apparu pour la première fois dans les années 1990, a été utilisé avec succès durant ces deux dernières décennies dans de nombreuses applications. Cependant, en toxicologie clinique et forensique (incluant par exemple la lutte anti-dopage ou les analyses post-mortem), son usage reste très limité et confiné à des applications spécifiques. En effet, la chromatographie gazeuse (*gas chromatography*, GC) ainsi que la chromatographie liquide (*liquid chromatography*, LC) sont les techniques les plus utilisées, que ce soit dans les approches de criblage générique ou lors de la quantification de composés d'intérêt. Ces deux techniques, particulièrement lorsqu'elles sont couplées à la MS, sont largement utilisées en raison de leurs nombreux avantages. De par son orthogonalité dans le principe de séparation, le couplage CE-MS permet d'offrir une technique alternative de choix dans les applications où la chromatographie montre ses limites (par exemple lorsque le volume d'échantillon est très faible, dans le cas de séparations énantiosélectives, ou dans le cas de composés très polaires), ou en addition à cette dernière, puisqu'une analyse orthogonale de confirmation est requise lorsqu'un échantillon criblé est identifié positif.

Cependant, la CE-MS reste rarement utilisée en toxicologie clinique et forensique. Ceci peut premièrement s'expliquer par un manque de connaissance du personnel de laboratoire quant à son utilisation. Certains ajustements doivent être effectués en amont de la séparation afin de garantir des analyses stables et répétables (par exemple, positionnement du capillaire dans la source ESI, conditionnement du capillaire, etc.). La répétabilité des temps de migration est également critique et souvent considérée significativement inférieure à celle observée en chromatographie. De plus, malgré l'amélioration importante de sensibilité offerte par le couplage avec la MS, le couplage CE-MS n'est souvent pas assez performant pour déterminer les très faibles concentrations attendues dans les échantillons biologiques tels que l'urine, ceci s'expliquant par les très faibles volumes injectés de l'ordre du nanolitre. Dans le but de pouvoir augmenter les performances du couplage CE-MS, incluant principalement la sensibilité, mais également la répétabilité, la rapidité, et la sélectivité, le procédé analytique peut être considéré dans sa totalité et des améliorations à chaque étape du processus analytique (préparation de l'échantillon, séparation, ionisation, détection) peuvent être envisagées. L'augmentation de la sensibilité analytique permet également d'améliorer la sensibilité diagnostique lors de tests de criblages, ceci dans le but d'éviter un nombre trop élevés d'échantillons présumés faux-négatifs.

Cette thèse a pour but de montrer les possibilités d'implémenter avec succès la CE-MS en toxicologie clinique et forensique grâce à des améliorations significatives proposées pour l'ensemble du procédé

analytique. La mise en œuvre d'une préparation d'échantillon permettant une préconcentration élevée peut être envisagée dans les cas où les composés à analyser sont plus ou moins connus et attendus, aboutissant ainsi à des sensibilités augmentées d'un facteur 100, voire plus. Dans ce travail, les techniques de préparation d'échantillon miniaturisées (microextractions) ont été considérées afin de rendre négligeable l'impact de la procédure analytique sur l'environnement. En raison des faibles volumes de solvants utilisés non seulement pour l'extraction mais également lors de l'analyse par CE-MS, les méthodes développées sont en accord avec les principes de la chimie verte. Le développement d'une méthode d'extraction basée sur la technique dite de microextraction dispersive liquide-liquide a abouti à la détection d'un grand nombre de drogues illicites et leurs métabolites respectifs avec des limites de détection situées en deçà du domaine du ng/mL.

Une préparation d'échantillon n'est parfois pas possible en raison de l'urgence de l'analyse ou lors de criblages à large spectre incluant un grand nombre de composés pouvant présenter des propriétés physico-chimiques très différentes. Dans ce cas, une préconcentration en ligne peut être mise en œuvre. En CE, les techniques de préconcentration d'échantillon en ligne sont basées sur la différence de migrations des composés au sein de milieux présentant des conductivités différentes. Dans cette étude, une méthode de criblage multi-composés a été développée, basée sur l'injection d'un large volume d'échantillon grâce à une technique de préconcentration reposant sur les différences de pH entre l'électrolyte de séparation et l'échantillon injecté. Aucune préparation d'échantillon n'a été requise et l'urine a simplement été diluée et acidifiée avant injection. Dans le but d'augmenter la répétabilité des analyses, un revêtement de capillaire a été proposé, permettant non seulement de très faibles variations dans les temps de migration mais également de diminuer le temps d'analyse. La méthode de criblage développée impliquant l'analyse par CE couplée à un analyseur de masse à temps de vol a permis d'obtenir des limites de détection dans le domaine du ng/mL en un temps d'analyse restreint (inférieur à 10 min). Une méthode de quantification a également été proposée en couplant la CE avec un analyseur de type triple quadrupole. La méthode a été validée pour deux composés modèles selon les procédures et recommandations officielles. Le domaine de concentrations validé montre l'application de la CE-MS pour la quantification de drogues d'abus dans des échantillons urinaires.

La toxicologie est un domaine qui inclut non seulement les composés de faibles poids moléculaires, mais également les protéines, avec par exemple la détermination de la transferrine désialylée dans le cadre du suivi d'alcoolisme chronique. La méthode analytique utilisée en routine (CE-UV) présentant quelques limitations en termes de sensibilité et sélectivité, le couplage CE-MS a été envisagé. Cette étude a mis en évidence les difficultés rencontrées lors du transfert de méthode en raison de l'adsorption de la transferrine à la paroi du capillaire ainsi que le manque d'efficacité du processus d'ionisation.

Ces diverses études illustrent donc les améliorations possibles qui peuvent être obtenues en optimisant chaque étape de la procédure analytique, permettant ainsi d'envisager le couplage CE-MS comme un outil analytique compétitif et utilisable en parallèle de la GC-MS et LC-MS dans diverses applications en lien avec la toxicologie clinique ou forensique.

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Chapter I.

Chapter I. Introduction

1 Toxicology

1.1 History

Poisoning has a history that dates back to the earliest times. At first used by old civilizations for hunting purposes or in warfare, poisons became over the time more sophisticated and were used to a larger extent. Numerous deliberate or accidental poisonings have been reported over the centuries. Socrate's famous death in 399 BC as a result of hemlock ingestion and genuinely related in *Phedon* remains one of the main foundations of modern philosophy. According to the legend, Cleopatra, last pharaoh of Ancient Egypt, died after having deliberately induced an aspis' bite on her breast. The Roman era is also associated to an extensive use of poisons by Roman emperors, who used to hide few amounts of cyanide at the dinner table to get rid of undesired opponents. Mithridates the Great was so frightened of being poisoned that he developed his own acquired immunity-like strategy by taking every day *sub*-lethal doses of substances as diverse as opium, cinnamon, or fennel seeds. Meantime, Dioscorides drafted the five volumes of *De Materia Medica*, the very first precursor of modern pharmacopeias, containing complete descriptions of *ca.* 500 plants with their medical effects on humans.

It was not until the Renaissance that the initial concepts of toxicology emerged with the innovative work of Paracelsus (1493 – 1541), whose quote s*ola dosis facit venenum* became a significant find at the time which is still valid today: "all substances are poisons, there is none which is not a poison; the right dose differentiates a poison from a remedy". Paracelsus was the first to recognize the importance of the dose-response relationship [1-4].

The foundations of modern toxicology were laid by Matthieu Joseph Bonaventure Orfila, a Spanish physician working in Paris, in the early nineteenth century. Experimenting that the dissolution of arsenic acid in a liquid would impede its detection with the existing methods, he developed new techniques and published in 1814 his *Traité des poisons, tirés des règnes minéral, végétal et animal, ou toxicologie générale*, the first book exclusively devoted to toxicology which described the physiological effects of numerous poisons (*e.g.*, stain, zinc, silver, gold, or bismuth preparations) and some novel procedures of identification [5].

1.2 Definitions

Toxicology can be defined as the branch of science that deals with poisons (toxicants), *i.e.*, any substance that causes a harmful effect when deliberately or accidentally administered to a living organism under specific conditions of exposure [1]. Toxicology is concerned with the physico-chemical properties of poisons, as well as their exposure, distribution, metabolism, and excretion. Qualitative and quantitative aspects step in the definition of poison. A compound can be toxic to one species or genetic strain, while being harmless to another. As an example, carbon tetrachloride is a potent hepatotoxicant in many species but is harmless to chicken. Moreover, compounds can be toxic combined with another one but without any negative effect alone [1]. Quantitative aspects are

illustrated with the dose-response relationship, as alleged by Paracelsus. Substances can be harmful at a defined dose but without any toxic effect at lower doses. Between these two limits, there is a wide range of effects ranging from long-term chronic toxicity to immediate death *via* acute intoxication. For example, huge amounts of water swallowed in reduced laps of time lead to a potentially lethal intoxication; metals are essential to living organisms at an appropriate dose but are toxic at higher doses [1]. Substances are defined by their *lethal dose* (LD), related to mortality, *toxic dose* (TD), where the measured response is a serious adverse effect other than lethality (*e.g.*, organ injury, coma), and *effective dose* (ED), corresponding to a therapeutic effect when existing. LD_{50} represents the dose that produces lethality in 50% of the tested organisms. Substances orally administered with LD_{50} lower than 5 mg/kg are considered supertoxic for humans, while being slightly or practically non-toxic at LD_{50} higher than 5000 mg/kg [3]. For therapeutic drugs, the difference in dose between the effective and the toxic dose represents the margin of safety. LD_{50}, TD_{50}, and ED_{50} are dependent on the route of administration of the toxicant, the potential interactions with other chemicals, as well as the gender, age, pharmacogenetic characteristics, and health status of the living organism [3].

Analytical toxicology focuses on the detection, identification, and quantitation of toxicologically relevant substances (poisons, xenobiotics, or metabolites) in biological or environmental samples, and includes three subspecialties: environmental, clinical, and forensic toxicology [1,3,6]. **Fig. 1.1** presents the subspecialties and their related disciplines involved in analytical toxicology. Environmental toxicology, which is concerned by the chemical exposures that are incidentally encountered in the living environment [3], will not be addressed here.

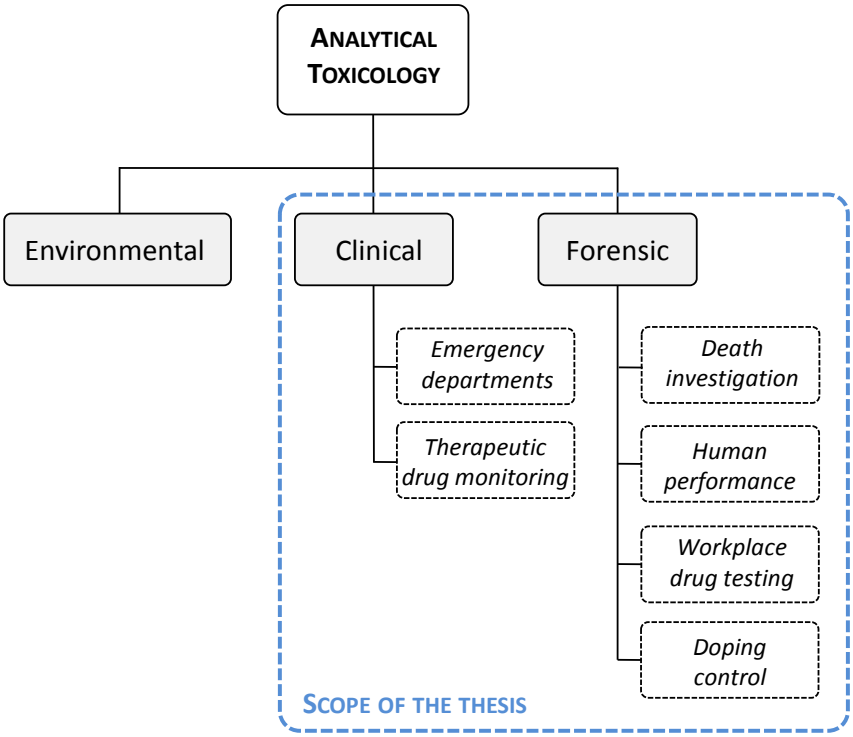

Figure 1.1. Analytical toxicology and related disciplines. The scope of the thesis is highlighted, focusing on clinical and forensic toxicology.

1.2.1 Clinical toxicology

Clinical toxicology is the discipline of toxicology concerned by the diagnosis and the treatment of poisoned or drug-affected patients, and studies the effects of chemicals intentionally or accidentally administered to a living organism [3,4,6]. This discipline encompasses a wide range of xenobiotics exposure. A xenobiotic (*ξένος*, foreigner, and *βίος*, life) is a substance present in an organism but extrinsic to the normal metabolism of that organism. Animal venoms, pollutants, solvents, pesticides, therapeutic agents, or drugs of abuse are numerous examples of xenobiotics. In living organisms, xenobiotics undergo biochemical modifications, the *metabolism*, to convert the parent compound into more readily hydrophilic excreted metabolites which as most frequently but not always inactive. The process is composed of two phases, *i.e.*, (i) *phase I reactions*, mainly catalyzed by cytochrome P450, with the addition of a reactive and polar group to the xenobiotic by oxidation, reduction, or hydrolysis; and (ii) *phase II reactions*, catalyzed by multiple enzymes such as sulfotransferases or glucuronosyltransferases, which consist of the conjugation of charged species (*e.g.*, sulfate or glucuronic acid) to the phase I metabolite. Phase II metabolites can then be excreted in urine to be eliminated from the organism.

Clinical toxicology also includes therapeutic drug monitoring (TDM), defined as the use of drug or metabolite monitoring in biological fluids as an aid to the management of a therapy [4]. TDM aims to individualize the dosage of a therapeutic agent according to its blood concentration for compounds presenting a concentration-effect relation. Suitable therapeutic candidates for TDM present a narrow margin of safety, as well as a high inter-individual and low intra-individual pharmacokinetic variability (*e.g.*, lithium, antiretroviral drugs, digoxin, aminoglycosides, immunosuppressants, *etc.*) [7-9].

Swiftness is a key factor in the management of poisoning due to the serious vital risks for the patient. In case of acute poisoning, which in three quarters of the cases results from an oral ingestion [10], the emergency approach aims to detect or exclude a potential intoxication, evaluate its seriousness and prognosis, and eventually select the adapted treatment. Handling a poisoned patient involves in a first instance an anamnesis and the systematic evaluation of clinical signs and symptoms, *i.e.*, vital signs (heart rate, temperature, and blood pressure), ocular findings (nystagmus, mydriasis), and mental status (coma, stupor, lethargy, delirium, *etc.*). These physical signs are referred to as *toxidromes* and their collection helps in narrowing the diagnosis [10-12]. Blood parameters, *e.g.*, glucose, blood gases, hemoglobin, or coagulation, can also evaluated with point-of-care tests (POCT), which give an instant result at the site of the patient [13-16]. All these investigations are performed in parallel to initial supportive life-saving measures, including airways, breathing, circulation assistance, intravenous saline solution infusion, dextrose administration, gastrointestinal decontamination, or endotracheal intubation [10]. Some typical toxidromes also involve the administration of specific antidotes (*e.g.*, naloxone, flumazenil) which help both in the diagnosis and the prognosis of the poisoning [17,18].

In emergency departments, vital interventions are usually carried out within the first minutes of care without the necessity of laboratory assays. The clinical analysis remains most frequently considered as more significant than a toxicological analysis; in most of the cases, the evaluation of toxidromes in parallel to life-saving measures is sufficient in the management of a poisoned patient. This is

particularly the case for mild form of intoxication with prompt positive evolution or when the management and prognosis are not affected by laboratory results [10,19].

1.2.2 Forensic toxicology

Forensic toxicology is concerned by the application of analytical toxicology in situations that may have medico-legal consequences and where the results are likely to be used in a court of law. Four disciplines are distinguished in forensic toxicology, *i.e.*, death investigation toxicology, human performance toxicology, workplace drug testing, and doping control [6,20].

1.2.2.1 Death investigation toxicology

Death investigation toxicology, previously referred to as *post-mortem toxicology*, represents the most obvious discipline of forensic toxicology and concerns the analysis of post-mortem samples to establish the role of xenobiotics in the cause and process of death, supporting the work of medical examiners and coroners in autopsy findings. Reliable identification and accurate quantitation of the substance(s) which caused the death are required due to the legal implications.

Numerous additional challenges that are not significant in other forensic disciplines are encountered in death investigation toxicology. The presence of ethical and legal environment implies that the toxicological investigations are determined by the coroner or the court with similar responsibility in jurisdiction. The information about signs and symptoms before death is usually missing, making a comprehensive toxicological analysis on a broad range of compounds compulsory. The analysis is often complicated by the condition (putrefaction) or the type of specimens. Post-mortem redistribution of substances due to the disruption of cellular membranes can increase the blood concentration of some drugs. Pharmacokinetic and pharmacodynamic data on illicit drugs are relatively poor due to ethical and regulatory provisions, leading to difficult interpretation of drug concentrations, already complicated by missing information about route of administration, number of doses, *etc.* Finally, drugs present in post-mortem samples are prone to instability and rapid *in situ* bioconversion. Therefore, all these aspects show the challenges not only in terms of sample collection and analysis but also in data interpretation [6,21-24].

1.2.2.2 Human performance toxicology

Human performance toxicology, also referred to as *behavioral toxicology*, concerns the analysis of ante-mortem specimens to evaluate the effects of drugs and alcohol on human performance (impairment or enhancement) and behavior, as well as their medico-legal consequences. It includes drug- or alcohol-impaired driving, drug-facilitated crime and sexual assaults, vehicular assault and homicide, as well as aircraft, motor vehicle, and maritime collision investigations [6,20,25]. Blood is the preferred specimen as a dose-effect relationship is assumed between the drug or ethanol blood concentration and behavioral changes. Illicit drugs, *e.g.*, heroin, as well as misused therapeutic antidepressants, anti-anxiety medications, or myorelaxants show significant behavioral effects. Alcohol is frequently associated with inappropriate socially or driving behavior. It presents the advantage that a typical behavior can be expected at a given blood alcohol content in case of acute

alcoholic influence, as summarized in **Table 1.1** [26,27]. This allows the national regulatory agencies to legislate in case of drunken driving with the definition of blood alcohol content cut-offs for impaired drivers.

Table 1.1. Stages of acute alcoholic influence and intoxication [28].

Blood alcohol content (g/dL)	Stage of alcoholic influence	Clinical signs and symptoms
0.01 – 0.05	Subclinical	Influence/effects usually not apparent or obvious Behavior nearly normal by ordinary observation Impairment detectable by special tests
0.03 – 0.12	Euphoria	Mild euphoria, sociability, talkativeness Increased self-confidence; decreased inhibitions Diminished attention, judgment, and control Some sensory-motor impairment Slowed information processing Loss of efficiency in critical performance tests
0.09 – 0.25	Excitement	Emotional instability; loss of critical judgment Impairment of perception, memory, and comprehension Decreased sensory response; increased reaction time Reduced visual acuity and peripheral vision; and slow glare recovery Sensory-motor incoordination; impaired balance; slurred speech; vomiting; drowsiness
0.18 – 0.30	Confusion	Disorientation, mental confusion; vertigo; dysphoria Exaggerated emotional states (fear, rage, grief, *etc.*) Disturbances of vision (diplopia, *etc.*) and of perception of color, form, motion, dimensions Increased pain threshold Increased muscular incoordination; staggering gait; ataxia Apathy, lethargy
0.25-0.40	Stupor	General inertia; approaching loss of motor functions Markedly decreased response to stimuli Marked muscular incoordination; inability to stand or walk Vomiting; incontinence of urine and feces Impaired consciousness; sleep or stupor
0.35 – 0.50	Coma	Complete unconsciousness; coma; anesthesia Depressed or abolished reflexes Subnormal temperature Impairment of circulation and respiration Possible death
≥ 0.45	Death	Death from respiratory arrest

1.2.2.3 Workplace drug testing

Forensic workplace drug testing (WDT) concerns the drug testing during pre-employment, random and periodic monitoring of employees, related to transfer or promotion, or “for-cause” situations [20]. First tests in the workplace environment appeared in 1971 in the U.S. and were motivated by concern about how the drugs of abuse affected the combat readiness of the armed forces [25]. Today, potential U.S. military recruits must undergo drug testing and are referred to civilian treatment programs if tested positive. In Switzerland, the Federal Department of Defense, Civil Protection and Sports recently adopted a zero tolerance policy to prevent the consumption, possession, and deal of illicit drugs during the whole military service. Each soldier has to endorse a declaration of relinquishment at the beginning of military service and will be shifted in case of positive testing [29].

In the private sector, an increased concern about the use of illicit drugs has been observed since the mid-1980s, and many industries began to implement their drug-testing programs. Much more used and regulated in the U.S., WDT is performed on a smaller scale in Europe and had to wait until the start of the 21st century to see an outbreak of interest and release of guidelines [30].

1.2.2.4 Doping control

Forensic drug testing is extended to anti-doping analysis for professional and amateur athletes. The first International Sport Federation that banned doping was the International Association of Athletics Federation (IAAF) in 1928. The International Olympic Committee (IOC) edited the first list of prohibited substances in the 1960s, and the first anti-doping tests were introduced at the winter and summer Olympic Games of 1968 in Grenoble and Mexico, respectively. Years of doping-related scandals urged the need for an independent international agency. The First World Conference on Doping in Sport which was held in Lausanne, Switzerland, under the initiative of the IOC led in November 1999 to the establishment of the World Anti-Doping Agency (WADA). The WADA is an international and independent agency equally sponsored by sport associations and governments [31-33] and aims to harmonize anti-doping policies, rules, and regulations within sport organizations and among public authorities [34].

The World Anti-Doping Code (*the Code*), adopted in 2003, is the core document providing the rules about testing, laboratories, Therapeutic Use Exemptions, roles and responsibilities of signatories, athletes, and governments; and the List of Prohibited Substances and Methods [35]. An extensive definition of doping is also proposed in the Code, *i.e.*, "the occurrence of one or more of the anti-doping rule violations". Anti-doping rule violations are constituted by (i) the presence of a prohibited substance or its metabolites or markers in an athlete's sample, (ii) the use or attempted use by an athlete of a prohibited substance or a prohibited method, (iii) the refusal or failure without compelling justification to sample collection, or avoidance of sample collection, (iv) the violation of applicable requirement regarding athlete availability for out-of-competition testing, (v) the tampering or attempted tempering with any part of doping control, (vi) the possession of prohibited substances and prohibited methods, (vii) the trafficking or attempted trafficking in any prohibited substance or prohibited method, and (viii) the administration or attempted administration to any athlete of any prohibited substance or prohibited method, or assisting, encouraging, aiding, abetting, covering up, or any type of complicity involving an anti-doping rule violation [35].

1.3 Summary

Although covering similar analytes of interest, clinical and forensic disciplines rely on different approaches. **Table 1.2** proposes a concise summary of the main differences between both disciplines in view of emergency of analysis, legal and medical consequences, and the analytical approach which will be discussed hereafter.

Table 1.2. Differences between clinical and forensic toxicology [6,20].

Endpoint	Clinical toxicology	Forensic toxicology
Subjects	Intoxicated or poisoned living patients, from minor to serious clinical status.	Deceased persons; employees or applicant; drug- or alcohol-impaired driver; professional or amateur athlete.
Eagerness	Hospital-based, emergency rooms environment; bedside testing. A rapid answer is needed to ensure/confirm efficient therapeutic regimen.	Chain of custody procedures; adequate time. Conscious collection of proper biosamples; post-mortem cases or no vital risks.
Analysis	Time constraints; focus on toxidromes evaluation in parallel to life-saving measures. Laboratory assays not systematically performed and relevant.	Comprehensive analysis with greater emphasis on specificity and accuracy in both identification and quantitation. Careful attention is paid to the use of orthogonal confirmatory procedures to ensure the reliability of results.
Jurisdiction	Little involvement in judicial matters.	Court proceedings; legal consequences.

2 Analytical strategy

Clinical and forensic laboratories have to fulfill international standard guidelines edited by the International Organization for Standardization (ISO) and the International Electrotechnical Commission (IEC) to be accredited. Forensic laboratories usually follow the ISO/IEC 17025:2005 *General Requirements for the Competence of Testing and Calibration Laboratories* to demonstrate their management system (*i.e.*, quality, administrative, and technical systems governing the operations of a laboratory), their technical competencies, and their ability to generate technically valid results [36]. Clinical laboratories meet accreditation to ISO/IEC 15189:2012 *Particular Requirements for Quality and Competence in Medical Laboratories*, addressing the qualifications and on-going competency of personnel, laboratory accommodation, equipment, reagents and supplies, pre-analytical and analytical factors, quality assurance considerations, and post-analytical factors. A particular emphasis is given to the place of clinicians, ethical practices, and patient care [37]. Both documents are based on the Quality Management System defined by ISO 9000:2008 guideline and mainly focus on management and technical requirements.

For questions related to the analytical strategy, the reference methods, or the threshold values, more specific information is available in the numerous laboratory guidelines and standards for clinical and forensic toxicology [38].

2.1 Laboratory guidelines and standards

A wide variety of national and international guidelines and standards has been published by government legislations or professional associations. They can be broadly applied to clinical and forensic disciplines, or can be specific to a field of application (*e.g.*, anti-doping, WDT, sexual assault cases, *etc.*). They are usually subject to regular updates in view of technical improvements or newly emerging drugs. An overview of relevant standards and guidelines used in clinical and forensic toxicology is presented in **Table 1.3**.

Table 1.3. Overview of standards and guidelines**.**

Organization	Guideline	Scope	References
AACB	Policies, procedures and guidelines for point-of-care testing	Clinical (POCT)	[39]
AACB/ASCEPT /RCPA/RACP	Mass or molar? Recommendations for reporting concentrations of therapeutic drugs	Clinical & forensic	[40]
AACT	Practice Guidelines on the treatment of methanol poisoning	Clinical	[41]
AS/NZS	4308:2008 Procedures for specimen collection and the detection and quantitation of drugs of abuse in urine	Forensic	[42]
CLSI	Urinalysis; Approved Guideline Analysis of Body Fluids in Clinical Chemistry; Approved Guideline Evaluation of Precisions Performance of Quantitative Measurement Methods; Approved Guideline Procedures for the Handling and Processing of Blood Specimens for Common Laboratory Tests; Approved Guideline Toxicology and Drug Testing in the Clinical Laboratory; Approved Guideline Gas Chromatography/Mass Spectrometry Confirmation of Drugs; Approved Guideline Mass Spectrometry in the Clinical Laboratory: General Principles and Guidance; Approved Guideline Expression of Measurement Uncertainty in Laboratory Medicine; Approved Guideline Evaluation of Matrix Effects; Approved Guideline	Clinical & forensic	[43]
CPDD	CPDD Guidelines	Forensic	[44]
DGKC/DGLM	The Quality of Diagnostic Samples. Recommendations of the working group on preanalytical variables	Clinical	[45]
EWDTS	European Laboratory Guidelines for Legally Defensible Workplace Drug Testing Guidelines for Legally Defensible Workplace Drug Testing: Specimen Collection Procedures Drug and Alcohol Testing in Hair: Collection and Analysis Guidelines for Oral Fluid	Forensic (WDT)	[46-50]
FDA	Guidance for Bioanalytical Method Validation	Clinical & forensic	[51]
FFLM	Recommendations for the collection of forensic specimens from complainants and suspects	Forensic	[52]
GTFCh	Guidelines for quality assurance in forensic-toxicological analyses Guidelines for quality assurance in forensic-chemical analyses of medical drugs and controlled substances Toxicological analysis in the context of determining brain death Validation of methods for toxicological analysis in the context of determining brain death Recommendation on units for reporting drug concentration	Forensic	[53,54]
GTFCh/DGLM/ DGCK	Guideline for the determination of alcohol in blood for forensic purposes The Haemolytic, Icteric and Lipemic Sample Recommendations regarding their Recognition and Prevention of Clinically Relevant Interferences	Forensic	[55,56]
ILAC	ILAC-G19:2002 Guidelines for Forensic Science Laboratories ILAC-G26:07/2012 Guidance for the Implementation of a medical Laboratory Accreditation System	Forensic Clinical	[57] [58]
IUPAC Commission of Toxicology	Sample collection guidelines for trace elements in blood and urine	Clinical & forensic	[59]
NACB	Recommendations for the Use of Laboratory Tests to Support Poisoned Patients which present to the Emergency Department	Clinical	[60-64]
NPIS/ACB	Laboratory analyses for poisoned patient: joint position paper	Clinical	[65]
NSC CAOD	Committee Handbook and Operating Rules	Forensic (DUID)	[66]
SAMHSA	Mandatory Guidelines for Federal Workplace Drug Testing Programs	Forensic (WDT)	[67,68]
SCDAT	Swiss Guidelines Committee for Drugs of Abuse Testing	Clinical & forensic	[69]
SFTA/SFBC/ STC/SRLF/ SFMU/CNBH	Recommandations pour la prescription, la réalisation et l'interprétation des examens de biologie médicale dans le cadre des intoxications graves	Clinical	[70]
SOFT/AAFS	Forensic Toxicology Laboratory Guidelines	Forensic	[71]
SOHT	Guidelines for drug testing in hair	Forensic	[72]
SWGTOX	Recommendations of the Research, Development, Testing, and Evaluation Committee	Forensic	[73]

Organization	Guideline	Scope	References
TIAFT	Systematic Toxicological Analysis: Laboratory Guidelines Systematic Toxicological Analysis: Recommendations on Sample Collection; Systematic Toxicological Analysis: Recommendations on Sample Preparation of Biological Specimens	Forensic	[74]
UKIAFT	Forensic toxicology laboratory guidelines	Forensic	[75]
WADA	World Anti-Doping Code Athlete Biological Passport Operating Guidelines	Forensic (anti-doping)	[35,76]

AACB, Australasian Association of Clinical Biochemists; AAFS, American Academy of Forensic Sciences; ACB, Association of Clinical Biochemists; ASCEPT, Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists; AACT, American Academy of Clinical Toxicology; AS/NZS, Australian/New Zealand Standard; CAOD, Committee on Alcohol and Other Drugs; CLSI, Clinical and Laboratory Standards Institute; CNBH, Collège National de Biochimie des Hôpitaux; CPDD, College on Problems of Drug Dependence; DGKC, Deutsche Gesellschaft für Klinische Chemie; DGLM, Deutsche Gesellschaft für Laboratoriumsmedizin; DUID, Driving under the influence of drugs; EWDTS, European Workplace Drug Testing Society; FFLM, Faculty of Forensic and Legal Medicine; GTFCh, Gesellschaft für Toxikologische und Forensische Chemie; ILAC, International Laboratory Accreditation Cooperation; IUPAC, International Union of Pure and Applied Chemistry; NACB, National Academy of Clinical Biochemistry; NPIS, National Poisons Information Service; NSC, National Safety Council; POCT, point-of-care tests; RACP, Royal Australasian College of Physicians; RCPA, Royal College of Pathologists of Australasia; SAMHSA, Substance Abuse and Mental Health Services Administration; SCDAT, Swiss Guidelines Committee for Drugs of Abuse Testing; SFBC, Société Française de Biologie Clinique; SFMU, Société Française de Médecine d'Urgence; SFTA, Société Française de Toxicologie Analytique; SOFT, Society of Forensic Toxicologists; SOHT, Society of Hair Testing; SRLF, Société de Réanimation de Langue Française; STC, Société de Toxicologie Clinique; SWGTOX, Scientific Working Group for Forensic Toxicology; TIAFT, The International Association of Forensic Toxicologists; UKIAFT, United Kingdom and Ireland Association of Forensic Toxicologists; WDT, workplace drug testing.

Each laboratory is responsible for selecting the adequate guideline(s) according to the area of activity and the legal and regulatory environment [38]. In order to help in this selection, an international group of researchers developed a generic instrument, the *Appraisal of Guidelines, Research and Evaluation* (AGREE) instrument, to evaluate the quality of clinical guidelines [77]. The AGREE instrument is a tool assessing the methodological rigor and transparency in which a guideline is edited. A new AGREE II instrument has been proposed in 2010, aiming at (i) assessing the quality of guidelines, (ii) providing a methodological strategy for the development of guidelines, and (iii) reporting what information ought to be presented in guidelines, and how [78-80].

2.2 Strategy

2.2.1 Clinical toxicology

In emergency departments, a comprehensive toxicological analysis (*i.e.*, determination, identification, and quantitation of xenobiotics) in case of an acute poisoned patient is *prima facie* not considered relevant or mandatory due to the following reasons [10,19,60,65,81,82]:

(i) The timeliness of reporting toxicological results, including the time of specimen receipt within the laboratory until the availability of results, is substantial and often inadequate in acute emergencies.

(ii) Performing recurrent toxicological analyses of high quality requires reliable laboratory equipment and trained clinicians and laboratory staffs. This is not ensured in every hospital with intensive care facilities, mainly due to the substantial associated costs, especially in case of out-of-hours solicitations.

(iii) The great majority of acute poisoned patients (≥ 95%) are rather symptomatic or do not require a specific treatment. They remain under observation in emergency room and recover completely without any serious complications.

(iv) Even having identified the cause of poisoning often leads to a poor impact for the patient who will *per se* recover without the need for specific antidote.

(v) The use of analytical techniques can lead to inaccurate data such as false-positive or false-negative samples, especially when more than one substance is involved. Immunoassays have some limitations in sensitivity, selectivity, and availability for some drug classes; many clinicians or physicians are not aware of these limitations, leading to potential wrong interpretation in absence of laboratory staff.

However, according to numerous relevant guidelines, including the National Poisons Information Service and Association of Clinical Biochemists (NPIS/ACB) in United Kingdom [65], the National Academy in Clinical Biochemistry (NACB) in the U.S. [60], the French multidisciplinary working group *Toxicologie et biologique clinique* [70], and the Swiss Guidelines Committee for Drugs of Abuse Testing (SCDAT) [69], laboratory assays are recommended in parallel to life-supporting measures in the following situations:

(i) In order to confirm the diagnosis of poisoning if there is any doubt, or in case of differential diagnosis.

(ii) When a significant impact on the patient management is observed, leading to further investigations, antidote administration, re-evaluation of the treatment, or stopping the treatment; and for assessing suitability for organ donation.

(iii) In case of brain death diagnosis, to ensure the absence of centrally-acting drugs before proceeding to withdrawing mechanical ventilation.

(iv) In particular clinical presentations such as unexplained coma, heart failure, or seizures.

(v) If the diagnosis has a high potential of legal involvement/consequences.

Few years ago, a consensus based on a two-tier toxicology testing was proposed by the NACB and the NPIS/ACB commissions [60,65]. The first tier (*Tier I*) includes the specific assays in serum/plasma that should be performed on a 24-h basis in all hospitals that admit acute poisoned patients. The NPIS/ACB commission recommends that the results should be available within a maximum of 2 h, or sooner if possible. Ideal timeframe for toxicology tests in NACB guidelines is less than 1 h from the time of the specimen receipt. The second group (*Tier II*) is composed of more comprehensive assays that can be important for patient management or may have clinical significance but that are not urgent and infrequently needed. They can be performed in supra-regional laboratories or centers during the next normal working day. This grading in the time limit set for the response was also very recently underscored in the 2012 recommendations of the French *Toxicologie et biologie clinique* commission. They defined three levels of delay in the response: (i) the level 1, corresponding to a delay of 30 – 60 min, which includes the assays that help clinicians in patient management, (ii) the level 2, representing a delay of 4 – 24 h and are applied to less frequent assays which aim to adapt the treatment or the initial diagnosis, and (iii) the level 3, corresponding to a delay of ≥ 24 h, which is applied in case of scientific or medico-legal purposes [70]. Level 1 and 2 are similar to the two-tier testing raised by UK and U.S. commissions. A similar priority status for obtaining results is proposed by the SCDAT with priority levels I, II, and III corresponding to available results within 3 h, 24 h, and few days, respectively [69].

Table 1.4 presents the targeted compounds whose quantitation in serum/plasma is recommended due to the impact on poisoning management (*Tier I toxicology testing*) [65,81].

Table 1.4. Tier I testing (quantitative specific assays).

Acetaminophen	Lithium
Anticonvulsants	Methemoglobin
Carboxyhemoglobin	Paraquat[1]
Digoxin	Salicylates
Ethanol	Theophylline
Iron	Phenobarbital

[1]: in urine, qualitative test only.

Paraquat is only listed in the NPIS/ACB guidelines; due to its rare prevalence in U.S., it was not considered by the NACB guidelines. Anticonvulsants were not listed in the NPIS/ACB guidelines of 2002, but will be added to the revised guidelines probably released this year. The quantitation of Tier I compounds is important due to the availability of reference data and decision criteria (*e.g.*, nomograms) for a potential treatment, as in case of acetaminophen overdose. Acetaminophen (paracetamol) is frequently used for suicide attempt and represents more than 100,000 cases of overdose *per* year in U.S. [83]. Over the last decade, an increased number of acetaminophen-intoxicated patients have been observed in Switzerland. Widely used for its analgesic effect, acetaminophen induces acute hepatic toxicity with doses higher than 100-125 mg/kg due to the formation of the toxic metabolite *N*-acetyl-*p*-benzoquinonimine (NAPQI). At usual therapeutic doses, NAPQI is usually rapidly detoxified by conjugating with glutathione. The clinical signs associated with acetaminophen toxicity are not specific during the initial phase, which justifies its presence in **Table 1.4**. Moreover, the determination of plasma concentration (≥ 4 h post-ingestion to ensure complete absorption) in relation with the time after the ingestion may inform about the type of treatment required [83-85]. The Rumack-Matthew nomogram, showed in **Fig. 1.2**, allows for an evaluation of hepatic toxicity risks in case of single acute ingestion.

Table 1.5 lists the compounds that are tested in serum/plasma samples during a *Tier II toxicology testing* [65].

Table 1.5. Tier II testing (broad-spectrum, infrequent assays).

Acetylcholinesterase	Paraquat[1]
Arsenic	Phenobarbital
Ethylene glycol	Phenytoin
Lead	Thallium[2]
Mercury	Thyroxine
Methanol	Toxicology screen
Methotrexate	

[1]: in serum, quantitative test.

[2]: will be added in the current revision of NPIS/ACB guidelines

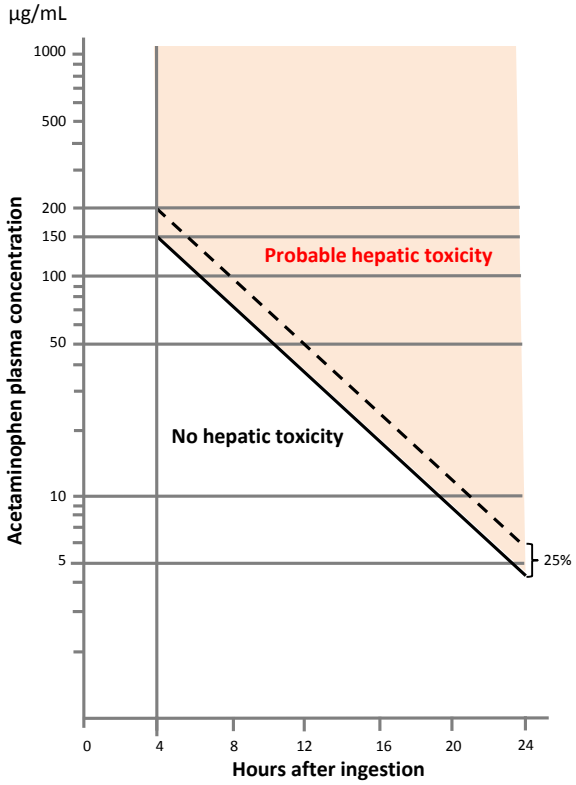

Figure 1.2. Rumack-Matthew nomogram. The nomogram represents the acetaminophen plasma concentration *versus* time post-ingestion. Dashed line expresses the Rumack-Matthew line (*toxicity line*), solid line the *treatment line* which is fixed 25% lower to include the analytical variability. In case of probable hepatic toxicity, *N*-acetylcysteine is administered and binds with NAPQI to inactivate it [85].

On the contrary of the NPIS/ACB commission, which recommends a *toxicology screen* (*e.g.*, screening of drugs of abuse) in the form of qualitative urine screening test only during the Tier II testing, the NACB has been promoting its implementation during the initial Tier I testing in certain conditions and only in presence of reliable analytical methodologies. The urine screening of drugs of abuse, including cocaine (COC), opiates, amphetamines, cannabis, and lysergic acid (LSD) has been also promoted in a first instance (*level I* or *level II*) in 2012 by the French commission. These assays require a careful clinical interpretation in accordance to the prevalence of the drug and the limitations of the techniques [60,70].

Over the past few years, significant improvements were carried out with the development of reliable, rapid, and highly sensitive and selective analytical techniques. Therefore, the current trend is towards implementing a systematic toxicological analysis (STA) in the previously outlined situations to correlate clinical signs with the presence of toxicants. STA involves high-throughput procedures and aims to detect a broad spectrum of relevant xenobiotics and to identify them unambiguously [74,86]. If required, this step can also be followed by a quantitative analysis of identified substances. In clinical fields, STA generally consists of a screening step performed with an immunoassay determination for a preliminary differentiation between presumptive positive and negative samples, and followed by a confirmatory analysis in case of positive result. Confirmation step should be performed with a new aliquot of the original specimen and with a different technique having a better specificity and at least equal sensitivity. This step is thus generally performed by chromatography combined with a selective and sensitive detector [70]. Depending on the requirements, a

chromatographic analysis may be directly used for the screening instead of the immunoassay determination while an orthogonal technique is used for the confirmation.

The implementation of STA and quantitation strategies has been also recently encouraged due to their importance in toxicological risk assessment, where very few or no human studies correlating the toxic concentration with corresponding clinical effect are available due to ethical issues [86,87]. Generating clinical toxicological case reports containing medical and toxicological laboratory results is thus closely recommended in evidence-based medicine. However, it is worth mentioning that the equipment required for comprehensive analysis workflows involves substantial investment costs and skilled clinicians and laboratory staffs; it is therefore still limited to important medical facilities.

2.2.2 Forensic toxicology

In contrast to the situation in emergency departments, the results in forensic toxicology may often be used in court proceedings. Therefore, the validity of the laboratory assays has to be ensured through a chain of custody, defined as the set of traceable records from the time of collection to the time of final disposal which document the chronologic disposition and condition of specimens. The chain of custody aims at answering questions regarding the localization of the samples or their possession. Lack of correct and intact chain of custody documentation may lead to inadmissible judgment of the toxicology testing in the court [20,88].

A comprehensive toxicological analysis is recommended in all forensic disciplines for the determination and quantitation of compounds. A STA procedure is usually first performed for identification purpose, either directed towards a class of drugs or covering a broad spectrum of compounds (*general unknown screening*). The initial screening step should always be confirmed whenever possible by a second analytical technique based on a different chemical principle to increase the reliability of the identification. During the screening step, the first recommended test should be a screen for the volatiles (*e.g.*, ethanol, methanol, or acetone) to avoid their depletion at each time the sample tube is opened. Compounds quantitation is carried out with a validated analytical method. The validation procedure includes numerous criteria to ensure the reliability and accuracy of the quantitation. The quantitation step may serve in certain conditions as an acceptable confirmation subsequent to the screening step [20,71,74,75]. **Fig. 1.3** summarizes the different steps of a forensic comprehensive toxicological analysis.

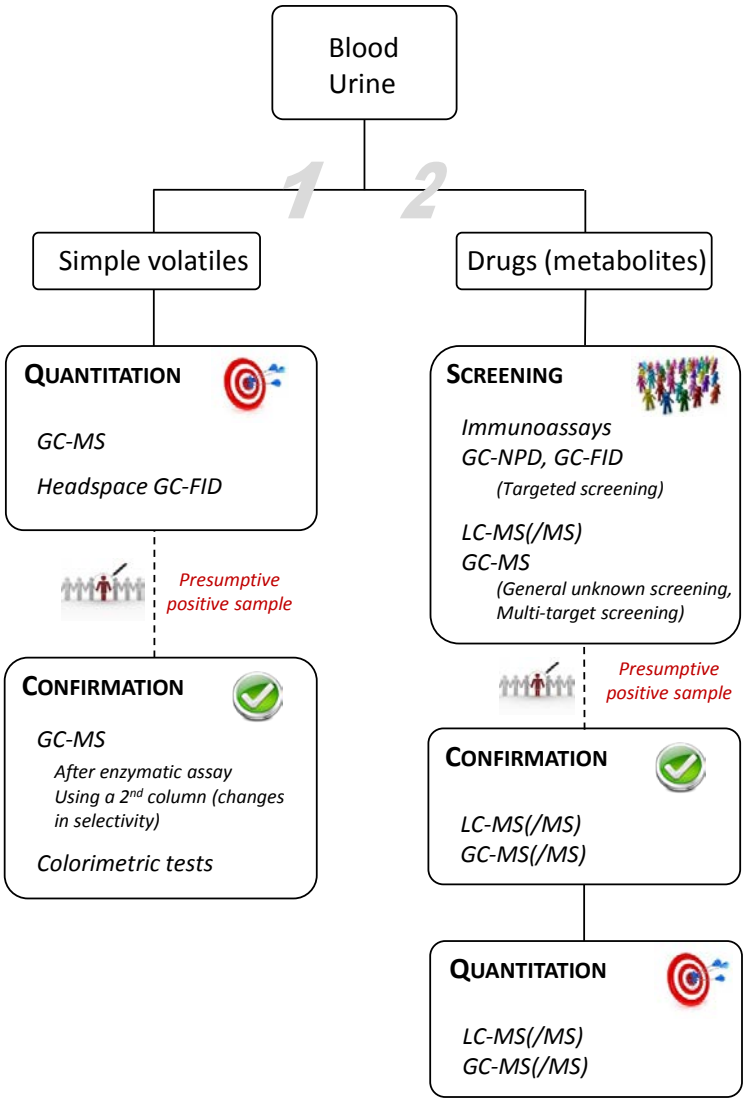

Figure 1.3. Comprehensive toxicological analysis. The recommended analytical techniques for each step of the procedure are presented in italic. FID, flame ionization detector; GC, gas chromatography; LC, liquid chromatography; MS, mass spectrometry; NPD, nitrogen-phosphorous detector; RT, retention time.

2.2.2.1 Death investigation toxicology

Relevant guidelines for death investigation toxicology were proposed in 2006 by the Society of Forensic Toxicologists (SOFT) with the Toxicology Section of the American Academy of Forensic Sciences (AAFS), which were reviewed and amended in 2010 by the United Kingdom and Ireland Association of Forensic Toxicologists (UKIAFT) for English and Irish application [71,75]. The guidelines describe relatively fully the requirements in terms of personal, standard operating procedures, samples and receiving, chain of custody, analytical procedures, quality assurance and quality control, review of data, results, and data interpretation. Regarding the samples, some recommendations are for example made about the amounts of specimen needed for a toxicological evaluation that should be collected at autopsy, as listed in **Table 1.6**.

Table 1.6. Recommended amounts of specimen by the SOFT/AAFS and UKIAFT guidelines [71,75].

Brain	50 g
Liver	50 g
Kidney	50 g
Cavity/heart blood	25 mL
Peripheral blood	10 mL
Vitreous humor	All available
Bile	All available
Urine	All available
Gastric content	All available
Hair	[1]

[1]: no recommended amount but cut from the vertex region of the scalp

Numerous recommendations are also proposed regarding the analytical procedures. Screening tests can be performed by immunoassays, color tests, or chromatographic methods with mass spectrometry (MS). The latter are highly recommended for confirmation purpose, but the rigorousness required for a confirmation depends on the importance of the analytical finding and case circumstances. If chromatography is used for screening and confirmation purposes, as for ethanol determination in blood, a system providing different selectivities (*e.g.*, different column chemistry, chemical derivatization, *etc.*) has to be used for the confirmation. A second immunoassay system cannot be used to confirm another immunoassay.

2.2.2.2 Human performance toxicology

The second scope of SOFT/AFFS and UKIAFT guidelines concerns the identification and quantitation of ethanol and other drugs for evaluation of their role in modifying human behavior. Although analytes are in many instances clearly specified, in contrast to post-mortem investigations, the same analytical approach is recommended.

Drug-facilitated sexual assault (DFSA) is a *sub*-discipline of human performance toxicology and is subject to dedicated guidelines regarding sample collection and analysis. More than 50 drugs are currently known or suspected to have been used in DFSA, *e.g.*, ethanol, benzodiazepines (BZD), amphetamines, dissociative anesthetics, or γ-hydroxybutyric acid (GHB), and have to be considered during the toxicological analysis [89]. These drugs are often rapidly absorbed, metabolized, and eliminated in less than 72 h; and lead to an alteration of consciousness and orientation with drowsiness, confusion, dizziness, and impaired memory and judgment, thereby rendering the analysis challenging and urgent. Numerous recommendations have been made for specimen collection, encouraging the sampling of blood (10 mL), urine, and hair, as well as mouth, skin, or vulval swab. Blood should be sampled within 72 h after the DFSA. Urine samples, having a longer detection time window, should be sampled within 96-120 h. Hair samples are important particularly if there is a delay in disclosing the incident and can be collected up to 6 months after it [52,72,89].

2.2.2.3 Workplace drug testing

First U.S. guidelines for drug-testing programs were edited in 1988 by the National Institute on Drug Abuse of the Federal Department of Health and Human Services, today known as the Substance Abuse and Mental Health Services Administration (SAMHSA) guidelines, updated in 2008 and corrected in 2010 [67,68]. In Europe, WDT is framed by the European Workplace Drug Testing Society (EWDTS) which edited the first European Laboratory Guidelines for Legally Defensible Workplace Drug Testing in 2002 [50]. Due to its ease of collection and testing, as well as the relatively high concentration of main compounds and metabolites, urine is the specimen of choice recommended by the guidelines for drug testing. Urine collection and transport are clearly defined in the guidelines to ensure privacy for donors and avoid tempting potential adulteration, *e.g.*, urine dilution, substitution of the sample, addition of substances, or use of detoxification kits available on the market [90]. The analytical strategy comprises an initial screening step by immunoassays followed by a confirmation with gas chromatography – mass spectrometry (GC-MS) for the determination of five major classes of drugs of abuse and their metabolites, *i.e.*, amphetamines, cannabinoids, COC, opiates, and phencyclidine [25,90-92]. The SAMSHA guidelines established specified cut-off levels for these compounds to define a positive result. **Table 1.7** presents the threshold concentrations for targeted analytes in immunoassay and GC-MS determinations.

Table 1.7. SAMSHA cut-off values [67].

Initial tested substance	Initial test cut-off level (immunoassay)	Confirmatory test analyte	Confirmatory test cut-off level (GC-MS)
Marijuana metabolites	50 ng/mL	Δ-9-tetrahydrocannabinol-9-carboxylic acid	15 ng/mL
COC metabolites	150 ng/mL	Benzoylecgonine	100 ng/mL
Opiate metabolites	2000 ng/mL	Codeine Morphine	2000 ng/mL 2000 ng/mL
6-Acetylmorphine	10 ng/mL	6-Acetylmorphine	10 ng/mL
Phencyclidine	25 ng/mL	Phencyclidine	25 ng/mL
Amphetamines	500 ng/mL	Amphetamine Methamphetamine	250 ng/mL 250 ng/mL
MDMA	500 ng/mL	MDMA MDA MDEA	250 ng/mL 250 ng/mL 250 ng/mL

Cut-off values were selected to help eliminating false-positive results; values below these cut-offs are reported as negative [90,91]. Based on these cut-off values, screening and confirmatory analyses are usually sufficient and an exhaustive quantitation is not required.

2.2.2.4 Doping control

Prohibited substances and methods are defined in the Prohibited List edited by the WADA and is revised at least once a year [35]. In order to be included in the Prohibited List, a substance or a method has to fulfill at least two of the following medical or ethical criteria: (i) having a potential or proof of enhancing sports performance, (ii) showing an evidence of a potential or actual health risk

to the athlete, and/or (iii) its use violates the spirit of sport as described in the Code [31,35,93]. **Table 1.8** summarizes the prohibited substances and methods present in the latest List, as well as their time of prohibition (in- and/or out-of-competition) and the sports specificities.

Table 1.8. WADA List of prohibited substances and methods (2013) [31,93,94].

	Examples
Prohibited Substances	
Non-approved substances[1,3]	Any pharmacological substance with no current approval by any governmental regulatory health authority for human therapeutic use (*e.g.*, designer drugs).
Anabolic agents[1,3]	Anabolic androgenic steroids and other anabolic agents.
Peptide hormones, growth factors and related substances[1,3]	Erythropoiesis-stimulating agents, chorionic gonadotrophin, corticotrophins, growth hormone.
Beta-2 agonists[1,3]	All beta-2 agonists except inhaled salbutamol and formoterol under maximal doses with Therapeutic Use Exemption.
Hormone and metabolic modulators[1,3]	Aromatase inhibitors, selective estrogen receptor modulators, anti-estrogenic substances, agents modifying myostatin function(s), metabolic modulators.
Diuretic and other masking agents[1,3]	Diuretics, desmopressin, plasma expanders, probenecid, *etc.*
Stimulants[2,3]	Non-specified and specified stimulants
Narcotics[2,3]	Opiates
Cannabinoids[2,3]	Natural or synthetic Δ-9-tetrahydrocannabinol and cannabimimetics
Glucocorticosteroids[2,3]	Cortisone, hydrocortisone, prednisone, betamethasone, *etc.*
Alcohol[2,4]	
Beta-blockers[2,5 and 1,6]	Acebutolol, atenolol, carvedilol, celiprolol, metoprolol, propranolol, *etc.*
Prohibited Methods	
Manipulation of blood and blood components[1,3]	Administration or reintroduction of autologous, homologous, or heterologous blood; artificially enhancing the uptake, transport, or delivery of oxygen; intravascular manipulation of blood.
Chemical and physical manipulation[1,3]	Tampering (urine substitution and/or adulteration); intravenous infusions and/or injections of more than 50 mL per 6-hour period.
Gene doping[1,3]	Transfer of polymers of nucleic acids; use of normal or genetically modified cells.

[1]: In- and out-of-competition
[2]: In-competition
[3]: In all sports
[4]: Aeronautic, archery, automobile, karate, motorcycling, and powerboating
[5]: Automobile, billiards, darts, golf, and skiing/snowboarding
[6]: Archery and shooting

The simple presence of a prohibited substance or its metabolites or markers in the biological matrix constitutes an Adverse Analytical Finding. However, for some of the compounds referred to as *threshold substances*, a sample is considered positive only above a given threshold and a quantitative step is therefore required [95]. Threshold substances for example include 19-norandrosterone, salbutamol, glycerol, morphine, cathine, and some ephedrine derivatives. For non-threshold substances, a minimum routine detection and identification capability has been established by the WADA to ensure that all the WADA-accredited laboratories can report the presence of prohibited substances. The Minimum Required Performance Levels (MRPL) Technical Document provides the concentration of prohibited substances, metabolites, and markers that accredited laboratories shall

be able to routinely detect and identify [96]. MRPL of non-threshold substances in human urine are presented in **Table 1.9**.

Table 1.9. MRPL for detection of non-threshold prohibited substances in human urine [96].

Prohibited class	Specific examples/exceptions	MRPL[1]
Exogenous anabolic androgenic steroids		5 ng/mL
	Dehydrochlormethyltestosterone	2 ng/mL
	Methandienone	2 ng/mL
	Methyltestosterone	2 ng/mL
	Stanozolol	2 ng/mL
Other anabolic agents	Clenbuterol	0.2 ng/mL
Beta-2 agonists (except salbutamol and formoterol)		20 ng/mL
Hormone antagonists and modulators	Aromatase inhibitors, SERMs and other anti-estrogenic substances	20 ng/mL
	Formestane	150 ng/mL
Diuretics and other masking agents		200 ng/mL
Stimulants		100 ng/mL
	Octopamine	1000 ng/mL
Narcotics		50 ng/mL
	Buprenorphine	5 ng/mL
	Fentanyl (and derivatives)	2 ng/mL
Cannabimimetics		1 ng/mL
Glucocorticosteroids		30 ng/mL
Beta-blockers		100 ng/mL

[1]: MRPL applies to the parent compounds, the metabolites, or the markers depending on each substance's biotransformation, excretion and/or stability in the sample

Over the last years, anti-doping has been facing numerous issues with the continuous emergence of new designer drugs produced by black-market laboratories, the use of market new drugs such as recombinant proteins and peptides that are similar or identical to those endogenously produced in the body, or the implementation of sophisticated doping protocols based on repeated microdoses administration [97]. Therefore, the early 2000s showed the emergence of indirect approaches, such as with the athlete biological passport (ABP), based on the detection of numerous endogenous biomarkers to assess the intake of prohibited substances. The principle of ABP relies on a longitudinal monitoring of selected variables to determine the probability of the data being physiological based on the athlete's previous values using Bayesian inference techniques [97-104]. Recent detection of abnormal values obtained during repeated, random, or targeted blood tests compared to the athlete's historical values can lead to doping suspicion. The WADA Athlete Biological Passport Operating Guidelines were first approved in 2009 and focus on a hematological module with the definition of 8 variables markers of blood doping, *i.e.*, hematocrit (HCT), hemoglobin (HGB), red blood cells count (RBC), percentage of reticulocyte (RET %), reticulocytes count (RET #), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) [105]. Two other calculated markers are OFF-score, a correlation between HGB and RET %, and abnormal blood profile score which combines HCT, HGB, RBC, RET %, MCV, MCH, and MCHC [106,107].

Today, an acceptable specificity and sufficient sensitivity are obtained with the hematological module of ABP [108]. Additional investigations are currently carried out to implement steroidal and

endocrinological modules [97,100]. The steroidal module aims to detect direct and indirect forms of doping with anabolic-androgenic steroids, and will be implemented in the very near future [109-112]. Endocrinological module related to growth factors (insulin growth factor-1 and growth hormone) is still in its early stages and requires further validation [97].

2.3 Systematic toxicological analysis

Except in WDT or in specific cases, *e.g.*, driving under the influence of drugs (DUID), where the toxicological analysis is based on a directed analysis to detect a single substance or a group of substances, a comprehensive strategy, the STA, is usually encouraged for the detection and unambiguous identification of all substances of toxicological relevance and their respective metabolites.

The scope of the detection stage should be stated prior to the STA to define the groups of relevant compounds for the given area of interest, in order to select the appropriate analytical techniques. Immunoassays are widely used for screening purposes due to their ease of use and speed. A presumed positive result is always confirmed with a more selective approach. Chromatographic techniques are recommended for the confirmation step; they are also increasingly considered in the initial screening instead of immunoassays due to better performance in terms of analytical selectivity and sensitivity as well as diagnostic sensitivity and specificity. State-of-the art screening approaches include GC-MS or liquid chromatography-mass spectrometry (LC-MS). In these approaches, *targeted screenings* (*e.g.*, in WDT) are typically performed with GC-MS or LC-MS in single reaction monitoring (SIM), or with GC-MS/MS or LC-MS/MS in selected reaction monitoring (SRM); while the *multi-target screenings* are performed with GC-MS in full-scan mode or LC-MS/MS with triple quadrupole or ion trap, and the *general unknown screenings* by GC-MS in full-scan mode or LC hyphenated to high resolution mass analyzers, such as time-of-flight (TOF) or hybrid analyzers [87].

In case of possible presence of toxicologically relevant compound(s), an unambiguous identification has to be established, obtaining only one suitable candidate matching to the detected feature. A single analytical method is often not sufficient to reach this unambiguous identification due to the large number of substances having close structural resemblances. Therefore, at least two analytical methods are usually required to exclude all possible candidates except one. It is worth mentioning that a positive *confirmation* test does not necessarily provide an unambiguous *identification*, even if both terms are often alternatively used. Confirmation has been defined by the presumption of the presence of a given substance in a sample based on initial tests or prior information, while identification does not make *a priori* presumptions based on initial tests or information [113]. Confirmation indicates that the test result is not against the presumption; unambiguous identification requires that all other relevant substances are excluded, leading to only one possible candidate (exclusion criterion).

2.3.1 Field of compounds investigation

Clinical and forensic toxicology involves the determination of several hundreds of compounds which differ from their physico-chemical (pKa, partition coefficient) and pharmacokinetic (absorption, distribution, metabolism, and excretion) properties. The broad range of compounds includes for

example metals, toxins, therapeutic drugs, or drugs of abuse. The latter are of major concern due to their high prevalence and the continuous emergence of new drugs on the illicit drug market. At least 85 million adults have used an illicit drug as some point in their life in the European Union, mostly cannabis (*ca.* 25% of Europe's adult population) [114]. Amongst the well-established psychoactive substances, an increasing number of new drugs mimicking their effects have been appearing over the last years at a relatively fast rate. It has been recently highlighted that every week a new substance has appeared in the European Union during the first half of 2013 [114]. COC, amphetamine, and ecstasy are still the most commonly used illicit stimulants in Europe but are followed by emerging drugs including piperazines, pyrrolidinophenones, and synthetic cathinones.

Table 1.10 provides a list based on current know-how of the relevant drugs of abuse and misused therapeutics available on the market as well as their relevant phase I and/or phase II metabolites. The pharmacokinetic properties of recent designer drugs, mainly synthetic tryptamines, some phenethylamines, and benzodifurans, have not been studied yet in humans and little information on their metabolism is available. All the drugs of abuse listed in **Table 1.10** as well as their relevant metabolites should be included in a comprehensive STA, particularly in death investigation toxicology.

Table 1.10. Drugs of abuse, misused therapeutics, and respective relevant metabolites [69,115-125]

Drugs of abuse	Relevant metabolites
Amphetamines	
Amphetamine[1]	Hydroxyamphetamine, norephedrine (phenylpropanolamine)
3,4-methylendioxymethamphetamine (MDMA, *Ecstasy, Adam*)	4-hydroxy-3-methoxymethamphetamine and glucuronide conjugates, MDA, 4-hydroxy-3-methoxyamphetamine
Methamphetamine[2]	Amphetamine, 4-hydroxyamphetamine
3,4-methylenedioxyamphetamine (MDA)	Dihydroxyamphetamine
3,4-methylenedioxyethylamphetamine (MDEA, MDE, *Eve*)	3,4-dihydroxyethylamphetamine, 4-hydroxy-3-methoxyethylamphetamine, MDA
N-methyl-benzodioxolyl-butanamine (MBDB, *Eden*)	Benzodioxolyl-butanamine, 1,2-dihydroxy-4-[2-(methylamino)butyl]benzene
Methylphenidate	Ritalinic acid, 6-oxo-ritalinic acid. For ethanol co-consumption: ethylphenidate
Fenfluramine	Norfenfluramine
Benfluorex	Norfenfluramine
4-methylthioamphetamine	Hydroxy metabolite and sulfoxide
4-para-methoxy-amphetamine	1-(p-methoxyphenyl)-propan-2-one, 1-(p-methoxyphenyl)-propan-2-one oxime, glucuronide and sulfate conjugates
4-para-methoxy-methamphetamine	4-hydroxy methamphetamine (pholedrine), 4'-hydroxy-3'-methoxymethamphetamine, glucuronide and sulfate conjugates
Tetrahydrobenzodifuranyl (*FLY*)	n.d.
Benzodifuranyl aminoalkane (*DragonFLY*)	n.d.
Bromo-benzodifuranyl-isopropylamine (*BromodragonFLY*)	n.d.
β-Keto-amphetamines (cathinones)	
Cathinone	Norephedrine, norpseudoephedrine
Methcathinone (ephedrone)	Ephedrine, pseudoephedrine
Methylmethcathinone (mephedrone)	Methcathinone (normephedrone), hydroxymethylmephedrone, hydroxynormephedrone and glucuronide conjugates
Methylone (bk-MDMA)	MDMA
Ethylone (bk-MDEA)	MDEA
Butylone (bk-MBDB)	MBDB
Barbiturates	
Pentobarbital	Hydroxy, carboxy, and glucuronide conjugates
Phenobarbital	Hydroxy, carboxy, and glucuronide conjugates
Secobarbital	Hydroxy, carboxy, and glucuronide conjugates
Benzodiazepines (BZD)	
1,4-BZD (diazepam, chlordiazepoxide)	Nordiazepam, oxazepam, and glucuronide conjugates
7-nitroBZD (flunitrazepam, nitrazepam)	*N*-acetyl-3-hydroxy-BZD, 7-amino-3-hydroxy-BZD, and glucuronide conjugates
TriazoloBZD (alprazolam, midazolam)	Hydroxy metabolites
Cannabinoids (marijuana, hashish)	
Δ-9-tetrahydrocannabinol (THC)	11-hydroxy-THC, 11-nor-THC-9-carboxylate, and respective glucuronide conjugates
Synthetic cannabinoids	
1-penthyl-3-(1-napthoyl)indole (*JWH-018*)	Hydroxylated metabolites and glucuronide conjugates
1-butyl-3-(1-naphthoyl)indole (*JWH-073*)	Hydroxylated metabolites and glucuronide conjugates
Cocaine	

Drugs of abuse	Relevant metabolites
Cocaine (COC)	Benzoylecgonine, ecgonine, norCOC, ecgonine methyl ester (methylecgonine, EME). For crack: anhydro ecgonine methyl ester (AEME); for ethanol co-consumption: cocaethylene
γ-hydroxybutyric acid	
γ-hydroxybutyric acid (GHB)	Unchanged GHB (< 5%), no metabolites
γ-butyrolactone	GHB
Butane-1,4-diol	GHB
Ketamine	
Ketamine	Norketamine, dehydronorketamine, and respective hydroxy- and glucuronide conjugates.
Methadone	
Methadone (MTD)	2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), 2-ethyl-5-methyl-3,3-diphenylpyrrolidine (EMDP), and respective glucuronide conjugates; methadol, normethadol
Methaqualone	
Methaqualone	3-hydroxymethaqualone, 2-hydroxymethyl-methaqualone, and glucuronide conjugates
Nicotine	
Nicotine	Trans-3'-hydroxycotinine, cotinine, nicotine-1'-*N*-oxyde, and glucuronide conjugates
Opiates and opioids	
Heroin	6-monoacetylmorphine (6-MAM), codeine, morphine
Codeine	Morphine, norcodeine, codeine-6-glucuronide
Dihydrocodeine	Dihydromorphine, dihydromorphine-6-glucuronide
Morphine	Morphine-6-O-glucuronide, morphine-3-O-glucuronide, normorphine
Hydromorphone (HMOR)	NorHMOR, hydromorphol, norhydromorphol, HMOR-3-glucuronide
Oxycodone	Noroxycodone, oxymorphone, noroxymorphone, 6-oxycodol, nor-6-oxycodol, and glucuronide conjugates
Oxymorphone	Noroxymorphone and glucuronide conjugates
Buprenorphine	Norbuprenorphine
Fentanyl	Norfentanyl
Tilidine	Nortilidine
Tramadol	*O*-desmethyltramadol
Pethidine (meperidine)	Normeperidine, normeperidinic acid
Propoxyphene	Norpropoxyphene
Phencyclidine	
Phencyclidine	4-phenyl-4-piperidinocyclohexanol, 1-(1-phenylcyclohexyl)–4-hydroxypiperidine, and glucuronide conjugates
N-(1-phenylcyclohexyl)-propanamine	Hydroxy- and N-dealkylated- metabolites
N-(1-phenylcyclohexyl)-3-methoxypropanamine	*N*-(1-phenylcyclohexyl)-3-hydroxypropanamine
N-(1-phenylcyclohexyl)-2-methoxyethanamine	*O*-deethyl-hydroxy- and *N*-dealkyl-hydroxy- metabolites
N-(1-phenylcyclohexyl)-2-ethoxyethanamine	*O*-deethyl-hydroxy- and *N*-dealkyl-hydroxy- metabolites
N-(1-phenylcyclohexyl)-3-ethoxypropanamine	*N*-(1-phenylcyclohexyl)-3-hydroxypropanamine
Phenethylamines (ring substituted)	
4-bromo-2,5-dimethoxyphenethylamine (2C-B)	4-bromo-2,5-dimethoxyphenylacetic acid, 4-bromo-2-hydroxy-5-methoxyphenylacetic acid
4-chloro-2,5-dimethoxyphenylethylamine (2C-C)	n.d.
2,5-dimethoxy-4-methylphenethylamine (2C-D)	*N*-acetyl metabolites and conjugates
2,5-dimethoxy-4-ethylphenethylamine (2C-E)	*O*-demethyl and N-acetyl metabolites and conjugates

Drugs of abuse	Relevant metabolites
2,5-dimethoxy-4-ethylthiophenethylamine (2C-T-2)	n.d.
4-iodo-2,5-dimethoxyphenethylamine (2C-I)	*O*-demethyl and *N*-acetyl metabolites and conjugates
4-propylthio-2,5-dimethoxyphenethylamine (2C-T-7)	n.d.
2,5-dimethoxy-4-nitro-β-phenethylamine (2C-N)	n.d.
Mescaline	3,4,5-trimethoxyphenylacetic acid, *N*-acetyl-β-(3,4,dimethoxy-5-hydroxyphenyl) ethylamine, *N*-acetylmescaline
2,5-dimethoxy-4-bromoamphetamine (DOB)	*O*-demethyl and deaminated metabolites and conjugates
2,5-dimethoxy-4-bromo-methamphetamine	*O*-demethyl and deaminated metabolites and conjugates
Piperazines	
N-benzylpiperazine (BZP)	4-hydroxy-3-methoxy-BZP and conjugates, piperazine, benzylamine
1-(3,4-methylenedioxybenzyl)-piperazine	4-hydroxy-3-methoxy-BZP, piperazine
(4-methoxyphenyl)-piperazine	Hydroxyphenylpiperazine glucuronide and sulfate
1-(3-trifluoromethylphenyl)-piperazine	4-hydroxy- metabolites and glucuronide/sulfate conjugates
1-(3-chlorophenyl)-piperazine (mCPP)[3]	Hydroxy-mCPP glucuronide and sulfate
Piperidines	
Pipradrol	n.d.
Desoxypipradrol	n.d.
Diphenylprolinol	n.d.
Pyrrolidinophenones	
α-Pyrrolidinopropiophenone	Hydroxyl- metabolite, cathinone,norephedrine
4’-methoxy-α-pyrrolidinopropiophenone	4'-hydroxy- and 4’-hydroxy-3'-methoxy- metabolites
3’,4’-methylenedioxy-α-pyrrolidinopropiophenone	4’-hydroxy-3'-methoxy- and deamidated metabolites
4’-methyl-α-pyrrolidinopropiophenone	2-oxo-4'-carboxy propiophenone, 4’-carboxybenzoic acid and glucuronide conjugates
3,4-methylenedioxypyrovalerone	Catechol and methyl-catechol pyrovalerone
Tryptamines	
Dimethyltryptamine	3-indolacetic acid
α-methyltryptamine	n.d.
α-ethyltryptamine	n.d.
N,N-diallyltryptamine	n.d.
Diethyltryptamine	n.d.
Di-isopropyltryptamine	n.d.
Dipropyltryptamine	n.d.
Psilocibine	Psilocine, 4-hydroxyindole-3-yl-acetate
Lysergic acid diethylamide	Nor-LSD, 2-oxo-3-hydroxy-LSD, and hydroxy-LSD and glucuronide conjugates
Volatile compounds	
Ethanol	Ethylglucuronide (EtG), ethylsulfate

[1]: some therapeutic or illicit drugs available on European market such as clobenzorex, mefenorex, selegiline, fenproporex, amphetaminil, prenylamine, and fenethylline are metabolized in amphetamine.

[2]: some therapeutic drugs available on European market such as benzphetamine, furfenorex, selegiline, and fencamine are metabolized in methamphetamine.

[3]: mCPP is a metabolite of trazodone, nefazodone, etoperidone, and mepiprazol.

n.d.: not determined

2.3.2 Biosamples

The choice of the biological specimen involved in STA relies on multiple criteria, such as (i) the sample availability and integrity, especially in the case of post-mortem analysis (potential putrefaction), (ii) the available sample amount, (iii) the type of sample collection, (iv) the gathered information by the toxicologist about the consumption/exposure, (v) the time of collection after the consumption/exposure, and (vi) the complexity of the sample (specimen pre-treatment, data interpretation).

Urine is the most widely used specimen for comprehensive screening. Blood, plasma, and serum are also often considered in numerous cases, in addition to or substituting urine screening. Other matrices such as hair and oral fluid have gained in importance over the last years, providing additional advantages and information. Biological specimens differ from each other by the detection times of xenobiotics and metabolites after the consumption, as highlighted in **Fig. 1.4**. Besides the matrix, the detection time depends on the dose, the preparation and route of administration, short-term *versus* long-term use, the physico-chemical properties of the xenobiotic and its metabolites, the detection limit of the analytical technique, the inter-individual variations in metabolism, and the pH in case of urine and oral fluid. Hair provides the longest detection times (up to 6 months), followed by urine (between 1.5 and 4 days, up to several days in case of chronic use), sweat, oral fluids (between 5 and 48 h), and blood (24-48 h) [91,126].

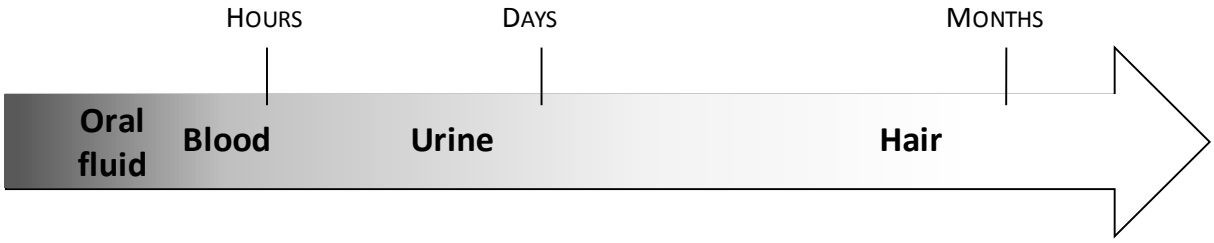

Figure 1.4. Averaged detection time windows for xenobiotics and metabolites in biosamples.

The pre-analytical step, including specimen collection and sample storage, are considered an important part of the whole analytical process. In post-mortem toxicology, a limited time should be observed between death (or finding of the victim) and specimen collection due to post-mortem redistribution [127]. Every specimen collection process should apply the following recommendations [128]:

(i) Ensure an appropriate labeling of the sample containers, including request number, name of the victim or other identifier, specimen type, anatomic place of collection, signature of the collector, and date and time of the collection. Proof of specimen integrity is promoted in post-mortem analysis (tamper-resistant stickers).

(ii) Collect the specimen with separate disposable or clean devices (scalpels, needles) for each specimen to avoid contamination.

(iii) Carefully select the containers to avoid both loss of analytes and introduction of contaminating substances. Glass is mandatory for volatile solvents, and preferred for other substances. Plastic containers can be used if potential contamination of the sample has been

excluded. Plastic must be carefully chosen to avoid cracking when frozen (*i.e.*, use polypropylene instead of polystyrene).

(iv) Fill the container as full as possible to minimize oxidative losses due to air trapped at its top, compounds volatilization, or salting-out effects of additives. It should be opened only when required for the analysis and when cold (4 °C).

(v) Handle the samples with care and always treat them as if they were infective (by human immunodeficiency virus, hepatitis B, tuberculosis).

Conditions and duration of handling and storage have also an important impact due to potential change in compounds concentration, *e.g.*, modification of endogenous steroid profiles in urine at room temperature due to microbial activity [129]. Specimens should be stored at an appropriate temperature (*i.e.*, 4 °C for short-term storage and -20 °C or -80 °C for more than a week), with an adequate preservative if required, and in a safe and suitable environment [128]. Blood, plasma, or serum samples are considered stable under these conditions; otherwise instability can be observed especially for compounds carrying ester moieties (COC, methylphenidate), sulfur atoms (phenothiazines), or other easily oxidized/reduced structures (nitro-BZD) [130]. Hair, nails, and dried-blood spot (DBS) specimens can be stored at room temperature as they have shown to be stable.

2.3.2.1 Biological specimens

2.3.2.1.1 Urine

Urine is the preferred specimen for screening procedure due to its ease of collection (except in renal failure or incontinence), the large volume of sample that can be collected, and the relatively high concentration of xenobiotics and/or their metabolites. Moreover, urine usually contains low amounts of proteins, lipids, and other high-molecular weight compounds due to the kidney glomerular filtration and thus requires minimal, if any, sample pre-treatment [91,128,131,132]. Xenobiotics and/or metabolites can be detected few hours after ingestion and up to several days. **Table 1.11** presents estimated detection times for some drugs of abuse in urine samples.

Urine is generally preferred for qualitative purposes and not frequently considered in quantitative procedures due to the very little correlation between urine and blood concentration as well as between concentration and clinical status. It can be collected as a spot sample (preferably in the early morning, when samples are the most concentrated) or as 24-h samples. Urine samples are prone to adulteration, especially in WDT or anti-doping control, even if appropriate measures are usually taken during the collection to avoid tempting sample manipulation. Creatinine concentration, pH, specific gravity, and temperature should be measured after urine collection to bring out any sample degradation or a potential exogenous modification, *e.g.*, urine dilution or addition of liquid soap, chlorine bleach, ammonia, hydrogen peroxide, vitamins, eye drops, or lemon juice [69,91,133]. The SCDAT, EWDTS, and SAMHSA guidelines have fixed some criteria to ensure the integrity of the sample, listed in **Table 1.12** [50,67,69].

Table 1.11. Detection times in urine samples of selected drugs of abuse [91].

Drug of abuse	Detection time
Amphetamines	
Amphetamine	48 h
Methamphetamine	48 h
Barbiturates	
Short-acting (pentobarbital)	24 h
Long-acting (phenobarbital)	3 weeks
BZD	
Short-acting (lorazepam)	3 days
Long-acting (diazepam)	30 days
COC metabolites	2 – 4 days
Marijuana	
Single use	3 days
Daily use	10 – 15 days
Long-term heavy smoker	> 30 days
Opioids	
Codeine	48 h
Heroine metabolites	48 h
HMOR	2 – 4 days
MTD	3 days
Morphine	48 – 72 h
Oxycodone	2 – 4 days
Propoxyphene	6 – 48 h
Phencyclidine	8 days

Table 1.12. Criteria of EWDTS, SCDAT, and SAMHSA to ensure the integrity of urine sample [38].

Adulteration	EWDTS	SCDAT	SAMHSA
Urine dilution			
Creatinine[1]	0.5 – 2 mM	0.4 – 1.8 mM	2 – 20 mg/dL
Specific gravity[1]	1.001 – 1.020	1.001 – 1.003	1.001 – 1.003
Sample substitution			
Creatinine	≤ 0.5 mM	< 0.4 mM	< 2 mg/dL
Specific gravity	Out of range 1.001 – 1.020	< 1.001	< 1.001
Sample adulteration			
Nitrite	> 500 mg/L	> 500 mg/L	> 500 mg/L
pH	< 3 or > 11	< 3 or > 11	< 3 or > 11
Other		Presence of endogenous or exogenous substances	Presence of endogenous or exogenous substances

[1]: reference values; urine is diluted if out of these ranges.

The WADA also specified in their 2004 Guidelines for Urine Sample Collection that the specific gravity should be greater than or equal to 1.005 using a refractometer or 1.010 with lab sticks, and the pH between 5 and 7 [134]. SAMHSA guidelines also differentiate urines with pH ≥ 3 and < 4.5 or ≥ 9 and < 11 which are considered invalid [67].

Renal tubules maintain blood pH by secreting hydrogen ions and weak organic acids and reabsorbing bicarbonate. Thus, urine has a usual range of pH between 4.5 and 8. It depends on the time of the day (lower pH values at night and in the early morning), diet (higher pH by vegetarian diet due to bicarbonate formation from fruits), health status, and medications. Although being sterile within the bladder, urine collection often involves a bacterial contamination due to the colonization of urethra by microorganisms. The bacterial decomposition of urea to ammonia and carbon dioxide leads to an increase in pH if the sample is not correctly stored [135]. This increase in pH can lead to compounds degradation, *e.g.*, hydrolysis of COC and 6-MAM and decreased solubility for phencyclidine. Right after the collection, temperature should be between 32 and 38 °C. In order to minimize potential degradation, urine should be rapidly frozen and stored as pre-aliquoted samples at -80 °C; and freeze-thaw cycles should be avoided [136]. Creatinine, uric acid, and urine osmolality are prone to significant changes with an increase in temperature [91,135]. An appropriate transport of the sample between point of collection and analytical laboratory is therefore crucial to ensure the sample integrity.

Transportation of doping control urine samples from the collection sites to the WADA-accredited laboratories is surprisingly performed at ambient temperature for convenience. A chemical stabilization mixture has been recently proposed to prevent the microbial growth, the hydrolysis of glucuronide and sulfate conjugates, or the tempting adulteration by proteolytic enzymes in urine doping control samples [137]. This mixture contains sodium azide, antibiotics and antimycotics, a protease inhibitor cocktail, a serine protease inhibitor, and pepsin and trypsin inhibitors. Nevertheless, the absence of the potential analytical interferences during STA procedures must still be proven.

2.3.2.1.2 Blood, plasma, and serum

Blood, plasma, and serum samples show the best correlation between compound concentration and pharmacologic effect. The vast majority of ante-mortem analyses are performed on plasma or serum samples, while whole blood is only used in specific cases (*i.e.*, determination of cyanide, lead, mercury, cyclosporine, or chlorthalidone associated to erythrocytes) [138]. Serum is issued from coagulated blood while plasma is obtained after centrifugation of anticoagulated blood by separating out the cellular constituents. Plasma lacks fibrinogen, prothrombin, and other clotting agents. EDTA, heparin, and citrate are conventionally used as anticoagulants and should be carefully selected depending on the planned assays. EDTA and citrate can chelate metals while lithium heparin cannot be used in case of lithium determination [128,139]. Plasma and serum should be separated from erythrocytes as soon as possible to avoid any enzymatic activity or redistribution of the analytes between cells and liquid [128]. Other additives such as sodium or potassium fluoride (preservative) and ascorbic acid (antioxidant) are usually required to ensure blood stability, especially in forensic toxicology.

Serum analysis is often preferred to plasma because (i) it produces less fibrin precipitate in case of freezing-thawing cycles; (ii) it is not subject to the influence of the anticoagulant on the analysis, the stability, or the protein binding; and (iii) no dilution of the sample is performed during the collection. On the contrary, plasma presents some advantages such as a larger volume and less risk of hemolysis [138].

Post-mortem analysis is also often carried out with whole blood which presents some differences with ante-mortem specimen, *i.e.*, (i) a higher viscosity, (ii) the presence of sedimented cells, (iii) the possible contamination with tissue fluids before collection, (iv) a lower pH (down to 5.5) due to the sharp decrease of pH immediately after death, (v) 60-90 % of water content, and (vi) a high degree of hemolysis [128].

2.3.2.1.3 Oral fluid

Oral fluid has the main advantage to be non-invasively and rapidly collected. It is composed of multiple secretions occurring in the oral cavity, including secretions from the salivary (parotid, sublingual, and submaxillary) glands, oral mucosa, gingival crevices, cellular debris, as well as expectorated bronchial or nasal secretions, and possible blood derivatives from oral wound. The term *saliva* covers the glandular secretions directly collected from the salivary glands [128,140-143].

The collection can be performed by spitting, draining, or suction; in these cases the relatively low-volume sample is rather viscous and the collection can involve some safety issues (transmission of infectious agents). More likely is oral fluid collected with commercial kits consisting in an absorbent pad/foam which is then squeezed onto the analytical device or added to diluents and mixed, providing a non-viscous fluid for the analysis [142]. Foodstuffs, beverages, or teeth brushing should be avoided before the collection. The sample collection is less prone to adulteration than urine because a second person can assist to the collection without infringing the donor privacy [141,142].

Oral fluid is mainly composed of water (99 %) and contains a low amount of proteins, *i.e.*, 0.3 % of mucins and 0.3 % of digestion enzymes. The usual pH is 6.8 but increases (up to 8) with the flow rate, which is typically at 0.05 mL/min when sleeping and up to 1 – 3 mL/min when chewing. Flow rate and pH are influenced by circadian rhythm, diseases, therapeutics drugs, hormonal changes, stress, or salivation stimuli (*e.g.*, lemon or citric acid) [128].

Parent drugs are generally the dominant compounds detected in oral fluid and have short detection times. Similarities between oral fluid and blood concentrations are observed due to partitioning (passive diffusion) with blood [144]. The relative concentration of compounds between oral fluid and blood depends on the ionization of the compounds at the respective pH values of the two fluids, as well as protein binding affinity. Acidic drugs usually have lower concentration in oral fluid than in blood, while basic xenobiotics are higher concentrated. For compounds with pKa 5.5 – 8.5, such as numerous drugs of abuse, the oral fluid:blood ratio varies depending on the pH of the former and the type of collection. Therefore, oral fluid is mainly recommended for qualitative purposes or with cut-off values [128]. In case of positive samples, the confirmation step is preferably performed in blood if available. The main applications of oral fluid testing are WDT and roadside drug testing, including drugs of abuse and ethanol, as well as TDM.

2.3.2.1.4 Hair

Due to its high content in proteins (65 to 95%), mainly keratin, hair has been traditionally the specimen of choice for the determination of chronic metal poisoning due to the covalent complexes formed by metals bound to sulfhydryl groups of keratin [128]. Nowadays, hair testing is increasingly used in conjunction with urine samples and is able to confirm long-term exposure to xenobiotics over

a period of several months. Hair is a strong matrix, stable at room temperature, easily handled and transported, hardly tampered during the collection, non-invasively collected, and it has a high resistance to decay in post-mortem cases [49,72,140,145,146]. In addition to proteins, hair is constituted by water, lipids, and minerals, and is surrounded by a rich capillary system. Scalp hair growth rate is usually 0.6 – 1.4 cm/month. Possible routes of xenobiotic incorporation to hair are (i) passive diffusion from blood capillaries at the root bulb, (ii) diffusion from sweat or sebum, and/or (iii) external contamination *via* passive exposure [72,128]. Hair should be cut from the posterior vertex region of the head, close to the scalp, where the average growth rate is constant and 85 % of the hair is in the anagen phase (active growing). Pubic, beard, or axillaries hair can be collected if head hair is not available. Cut hair sample is then aligned with the root end and secured with an aluminum foil prior to a placement in a paper envelope. Storage in plastic bags is avoided because of potential contamination, and since the plastic can induce the extraction of lipophilic substances [146].

Prior to sample preparation, hair is washed to remove any hair care product, sweat, sebum, as well as potential external contamination that can lead to wrong interpretation of the analytical result, which can also be obtained in case of bleaching, relaxing, or dyeing hair treatments. Even with an efficient washing method, it is recommended for COC or cannabinoids suspicion to detect at least one metabolite in hair, in order to avoid potential false-positive due to environmental contamination [146].

Some drugs having quite low incorporation rate into hair, due to poor melanin affinity of blood membrane permeability, trace concentrations have to be detected. Therefore, if a urine sample was found positive but hair sample negative, the negative finding should not overrule the positive urine result [147].

2.3.2.1.5 Alternative specimens

The large majority of forensic or clinical screening assays involve the analysis of urine and/or blood samples. In addition to oral fluid and hair testing, other alternatives matrices can be also considered in specific applications, *e.g.*, post-mortem analysis, neonatal exposure, *etc.*

Liver, bile, vitreous humor, and cerebrospinal fluid are likewise collected in death investigation analysis. Liver drug test results can supplement any blood toxicological data. Bile, cerebrospinal fluid, and vitreous humor are useful for qualitative analysis when urine is not available [24,128]. Both cerebrospinal fluids and vitreous humor contain very little proteins [128]. Gastric content can be also helpful to determine if oral ingestion occurred within hours of a death [23]. Finger- and toenails have already shown to be valuable specimen in long-term exposure (even potentially longer than hair) with rather high detectable concentrations. Nails share the same issues than hair, *e.g.*, external contamination or variation in xenobiotic accumulation, but can be interesting in case of lack of hair availability [128].

Meconium, placenta, cord blood, amniotic fluid, and breast milk are considered relevant specimens in cases of pre- or postnatal exposure to drugs [148,149]. Meconium (first fecal matter passed by a neonate) can provide valuable information regarding *in utero* long-term exposure, as it begins to form at approximately 12 weeks of gestation (onset of fetal swallowing of amniotic fluid) and

because of the accumulation of xenobiotics over several months of gestation. Collection of meconium is recommended within 48 – 72 h after the delivery [128,145]. Mammary glands have various carrier-mediated systems which help in the transport of drugs of abuse into milk. Human breast milk shows a lower pH and higher content in proteins and lipids compared to plasma, but the exact mechanisms involved for each drug remains not fully elucidated. Its composition changes over the postpartum weeks, leading to time-dependent variations of drugs excretion into milk [150]. The concentration of a xenobiotic not only depends on the dose and duration of consumption, but also on the amount of milk daily excreted, the mother's health, and her genotype [151]. Breast milk can be non-invasively and relatively easily collected; however, due to its high amount in proteins and lipids and the changing composition during the post-partum period, the analysis of drugs remains challenging [151,152].

Sweat analysis has also been considered in the monitoring of individuals in drug rehabilitation, leading to a prospective (rather than a retrospective) approach. Passive diffusion from blood into sweat glands as well as transdermal passage of drugs across the skin likely explain the incorporation of compounds in sweat [145]. Non-occlusive sweat patches worn during a long time (> 24 h, usually 7 days) provide cumulative measure of drug exposure, detecting both parent drug and metabolites. It is more adapted to qualitative approaches since it suffers from a large variation in production and difficulties in estimating the volume of the specimen [153].

2.3.2.2 Sample preparation

In case of immunoassay determination, urine or plasma/serum samples do not require any preliminary treatment and are directly used with the immunological test. For all the chromatographic-based techniques involved in STA procedure, urine and plasma/serum cannot be directly injected and require a sample pre-treatment. The latter should be carefully selected according to the specimen and the performance of the analytical procedure. Advances in analytical technologies often allow for a simple removal of interferents rather than a selective, cost-effective, and time-consuming extraction. A selective clean-up is also not possible in STA; therefore, many interferents are co-extracted, highlighting the importance of a powerful analytical technique to provide sufficient separation and accurate detection for a reliable identification of unknown substances.

2.3.2.2.1 Urine

The easiest urine pre-treatment which can be envisaged prior to LC analysis is a simple dilution of the sample, referred to as *dilute and shoot* approach, enabled due to the relatively low complexity of the matrix. Prior to the dilution, urine is generally centrifuged to remove materials in suspension (*e.g.*, cellular components) and filtrated on 0.45- or 0.22-µm cellulose filters. Dilution is performed with water or buffers, but organic solvents can also be used to simultaneously induce a protein precipitation (PP). On the contrary to plasma/serum samples, where deprotoneization is an essential step, urine generally presents a very low content of proteins (0.5 – 1 g/L) which does not require a PP. Nevertheless, proteinuria is a very common clinical abnormality which is caused by number of pathologic conditions affecting the kidney and the urinary tract [154]. Dilute and shoot approach with LC-MS is particularly well suited for STA due to the injection of the whole matrix without any

discrimination, but it can lead to ion suppression in MS with co-eluting interferents, and can only be used with highly sensitive detection techniques [136,155,156].

GC-based analysis generally involves a first step of phase II derivatives hydrolysis to convert them into more suitable compounds for GC analysis. Acidic hydrolysis with concentrated hydrochloric acid is rapid but not often considered due to a potential loss of compounds and artifacts formation under these harsh conditions. Mostly preferred is the enzymatic hydrolysis of the conjugated compounds with biocatalysts, *e.g.*, β-glucuronidases from *E. Coli* or *H. Pomatia*, and arylsulfatases [136,157,158]. The time required for enzymatic cleavage is rather long (from 4-h to overnight incubation) but can be significantly reduced with ultrasounds or microwave irradiation to less than an hour [159].

Only water-free samples can be injected in GC; therefore, a subsequent extraction step is required. In LC analysis, the *dilute and shoot* approach is not necessarily adapted and an extraction step may be mandatory prior the analysis.

Liquid-liquid extraction (LLE) is historically the oldest sample preparation and is still frequently used in STA due to its applicability to a broad range of compounds and its ease of use. Multiple organic solvents or mixtures can be used to extract the compounds in one or more steps at various sample pH values. Too lipophilic solvents or mixtures should be avoided not to miss rather polar phase I and phase II metabolites. Apolar solvents such as ethyl acetate or dichloromethane containing various proportions of miscible polar solvents such as isopropanol, methyl tert-butyl ether, or acetone have been described in the literature to ensure the extraction of a broad polarity range of compounds without extracting to many matrix interferents [157,160]. Some drawbacks of LLE are the emulsion formation, making difficult to isolate the extraction solvent, the difficulty in automation, and the use of large volumes of organic solvents that are environmentally unfriendly and lead to an extra-step of evaporation and reconstitution. The evaporation step needs special attention due to potential loss of some semi-volatile compounds, *e.g.*, amphetamines. Automated 96-well polypropylene plates have been proposed in the last decade for LLE but are prone to cross-contamination due to the close vicinity of the wells and the organic solvent used [155]. Supported liquid extraction (SLE), which derives from LLE procedure, can be used as an alternative extraction technique enabling a complete automation. The principle of SLE relies on the absorption of the aqueous phase onto wide-porous and chemically inert diatomaceous earth present in a column and partition of the compounds with the elution solvent flowing under gravity [161-163]. The solvent consumption is similar or even higher to LLE, except in the 96-well format. Liquid-based microextraction techniques tend to overcome the lack of automation, the emulsion formation, and the high solvent consumption thanks to the miniaturization of the procedure and are discussed in **Chapter II**.

Solid-phase extraction (SPE) has been receiving more attention in recent years due to the possible automation and miniaturization, as well as the numerous sorbents that are commercially available. A careful selection of the extraction sorbent(s) is mandatory to allow for a wide extraction of the compounds with good recoveries. Silica-based sorbents with C_{18} or C_8 modifications have shown satisfying results in terms of extraction recovery for a large range of compounds, as well as good clean-up efficiency; and were thus widely used in the past [156,160]. However, the use of silica sorbents is limited to a short range of pH values, leading to poor recoveries for polar, hydrophilic, and ionized compounds. The introduction of polymeric and mixed-mode materials led to significant

improvement in developing versatile SPE procedures [155,158,164,165]. Polymeric sorbents are stable over a wide range of pH and are less soluble in some solvents compared with silica-based material, allowing for their use with harsh conditions. Mixed-mode sorbents are usually composed of ion-exchange and reversed-phase functions which permit the sequential elution of acidic, neutral, basic, hydrophobic, and/or hydrophilic compounds with modification of pH and elution composition. The different eluates are then combined and generally evaporated and reconstituted in the appropriate injection solvent. Recent SPE sorbents such as restricted access materials (RAM) or monoliths can also be considered for versatile extraction. RAM enables the direct injection of the matrix after a fractionation into a protein matrix and the analytes fraction based on size-exclusion of macromolecules and extraction of analytes [163]. Semi-automated 96-well formats increase the extraction throughput while decreasing sample and solvent consumption. **Chapter II** presents another step to decrease solvent and sample consumption with the use of solid-based microextractions. On-line coupling of SPE to LC can also be considered to allow for a partial or total automation of the analytical process, a lower analytes loss, and an enhanced sensitivity due to the injection of the whole extract [166]. On-line SPE-LC is usually achieved through column switching configurations, as shown in **Fig. 1.5**.

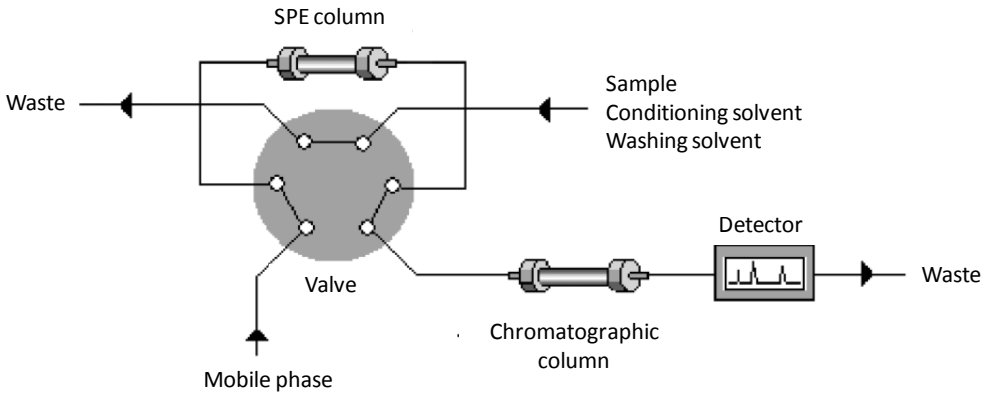

Figure 1.5. Typical configuration of on-line coupling of SPE to LC through column switching. Adapted from [166].

SPE still suffers from a possible batch-to-batch variability and comparable sorbents from different manufacturers may lead to different results [160,163,167]. The relatively high costs generated by the SPE cartridges and mainly by the 96-well plates where the wells are not all used at each time are evident drawbacks.

For GC analysis, an additional step is required with the derivatization of the compounds showing polar moieties to increase the volatility, especially for compounds carrying hydroxyl, ketones, amine, or carboxy groups. Decreasing the compounds polarity also improves the separation by minimizing the unwanted column adsorption, allowing for better peak shapes. Robust derivatization processes which lead to a single derivative without the formation of interferents are required. Silylation, acylation, alkylation, and formation of cyclic derivatives are most commonly used for the derivatization of extracted xenobiotics and metabolites [158,168].

Besides LLE and SPE, compounds can be also extracted in the context of urine collection and storage on a filter paper. This methodology, which derives from the DBS approach (presented in **Section**

2.3.2.2.2) is referred to as *dried-urine paper filter samples* or *dried urine spots* (DUS). DUS is particularly useful in newborn and infant screening, in which cases a filter paper is pressed onto the wet unsoiled diaper until it is thoroughly wet. Numerous procedures to extract the compounds of interest have been proposed, such as (i) direct extraction with organic solvents, *e.g.*, methanol and acetonitrile, (ii) pH adjustment with alkaline/acidic buffer prior to organic extraction, (iii) direct extraction with aqueous solution, or (iv) direct extraction with aqueous solution followed by LLE [169,170]. DUS has been already successfully applied for the determination of drugs of abuse [171]. DUS collection has also recently shown to inhibit the enzymatic degradation of phase II conjugates, *e.g.*, ethyl glucuronide, in case of bacterial contamination [172].

2.3.2.2.2 Blood, plasma, and serum

Due to their high content in proteins (60 – 80 g/L), blood, plasma, and serum samples cannot be directly injected and require a more intensive pre-treatment. Plasma/serum analysis rarely requires a cleavage of conjugates due to their low concentrations [165]. A PP is usually carried out prior to further extraction to avoid an important emulsion formation in LLE or clogging of the SPE sorbent [173,174]. Different approaches are considered to remove proteins in blood, such as the addition of organic solvent (acetonitrile, methanol, or acetone) or acids (perchloric acid, trichloroacetic acid) in a 2:1 or 3:1 ratio (*v/v*) with the sample [175,176]. Due to the relatively high volume of precipitating agent required, a highly sensitive analytical technique is mandatory. Moreover, low recoveries are usually obtained due to analyte co-precipitation during the procedure. After PP, isolation or centrifugation is carried out and the filtrate or supernatant may be directly injected with or without pH adjustment. However, significant ion suppression due to the presence of co-eluting matrix components is usually observed, thereby justifying an additional extraction step. These drawbacks can be overcome by using an alternative approach prior to LLE or SPE, *i.e.*, diluting plasma/serum sample with an appropriate buffer by a ratio 1:5 or 1:10, *v/v* (*e.g.*, 50 mM phosphate buffer at pH 7.4). The dilution decreases the viscosity and facilitates the flow through the SPE cartridge, but increases the amount or organic solvent required for LLE procedure.

Strategies in the selection of SPE sorbents and extracting solvents are the same as previously discussed for urine screening. Other SPE sorbents have also been developed to specifically remove phospholipids and proteins prior to the injection, providing a rapid and non-selective clean-up when no sample concentration is required [177].

Volatile compounds cannot be extracted with conventional LLE or SPE procedure. In this case, blood samples are diluted with alkaline/acidic buffer prior to headspace extraction and GC analysis. Another alternative is the use of solid-phase microextraction (SPME) in which the fiber is placed in the headspace compartment, leading to the adsorption of volatile and semi-volatile compounds which are then thermodesorbed in the injector and analyzed (*cf.* **Chapter II**).

The use of filter paper for collection and extraction of samples is prevalent for blood screening compared to urine analysis. DBS, which saw its very first appearance hundred years ago, is increasingly used for numerous applications in clinical and forensic STA. DBS can be obtained with a simple finger prick with resulting blood applied on a filter paper (directly with the finger pressed or with a precision capillary), providing significant advantages on conventional blood collection and analysis, such as (i) less invasive sample collection which does not require medical training, (ii) lower

volume, *i.e.*, *ca.* 20 µL instead of more than 0.5 mL, (iii) analyte stability at room temperature with reduced compounds hydrolysis, *e.g.*, COC and 6-MAM, (iv) improved safety due to the inactivation of some viruses that lose their infectious properties (*e.g.*, HIV-1), and (v) rapid collection in case of drugs with very short half-lives, such as GHB [178-180]. Compounds extraction is usually off-line performed using a mixture of aqueous solutions and organic solvents. An aqueous solution generally leads to a complete desorption, including analytes and matrix components. Contrariwise, the use of organic solvents induces an on-filter PP and a better clean-up, decreasing the ion suppression that can be observed in MS with co-extraction of matrix interferents [181,182]. Therefore, DBS is not only regarded as a support for sample collection, but also as an extraction tool due to the intrinsic properties of cellulose.

2.3.2.2.3 Alternatives matrices

A minimum sample pre-treatment is required for highly aqueous specimens, *e.g.*, sweat and oral fluids, which are usually simply diluted prior to the injection. Due to its high content in proteins, vitreous humor requires an additional PP step.

Hair is first systematically cut to the appropriate length with a suitable razor blade and the help of a graph paper. Hair decontamination should allow for the washing of external impurities without extracting the drugs of interest. Up to now, no consensus has been set for optimal decontamination but non-protic solvents, *e.g.*, dichloromethane or acetone, have shown to be advantageous. The first wash solution should be kept for further analyses. For screening purposes, the most adapted extraction procedure involves the use of methanol in an ultrasonic bath. Methanol penetrates the hair matrix, leading to hair swelling and drugs diffusion. The hair structure is degraded in the ultrasonic bath. A second step with LLE or SPE is generally recommended due to the high interferents present in the extract. Aqueous acids or buffer solutions can also be used to obtain cleaner extracts and better recoveries, but lead to compounds hydrolysis, *e.g.*, heroin, COC, and 6-MAM. Aqueous sodium hydroxide (1 M, 1 h at 80 °C) can also be considered with good extraction recoveries but compounds have to be stable under alkaline conditions [146,183].

For other solid or semi-solid specimens, such as liver, gastric content, or meconium, the specimen is first homogenized, followed by enzymatic digestion and filtration. Conventional extraction procedures are then used prior to their analysis.

Filter paper has also very recently shown to be fully applicable to other matrices than urine and blood, *e.g.*, vitreous humor, cerebrospinal fluid, and other tissues [184].

2.3.3 Immunoassays

Immunoassays provide numerous advantages such as simplicity of operation, minimal volume of sample required (10-200 µL), no specimen pre-treatment, and rapidity of the result. They have been widely used in emergency departments for screening purposes, where it is of utmost importance to obtain a response within few minutes. In many cases, an immunoassay determination represents the initial screening step of STA to quickly eliminate negative samples prior to chromatographic confirmation [131].

An immunoassay test relies on the specific competition of an antibody (Ab), namely immunoglobulins (Ig), for an antigen (Ag), namely the compound of interest. Ig are glycoproteins produced in living organisms by B-lymphocytes after immune system stimulation. Five classes of Ig are observed in mammalians according to the difference in their structure, *i.e.*, IgG, IgM, IgA, IgE, and IgD. The basic structure is composed of two identical heavy chains and two identical light chains. *N*-terminal ends of both heavy and light chains compose the variable region which serves as the Ag binding part of the molecule, referred to as the F(ab) (*fragment Ag binding*) region, providing the binding specificity. The arm containing only heavy chain is referred to as the F(c) (*fragment crystallizable*) region; both heavy chains are linked with disulfide bonds. The *epitope* is the area of the Ag that is recognized by an Ab. Van der Waals forces, hydrogen bonds, and ionic interactions are involved in the epitope-Ab binding. Abs used in immunoassays are produced in animals after immunization by the target Ag. Polyclonal antibodies are complex mixture of Abs with different specificities for epitopes and are usually obtained after immunization of sheep, goats, or rabbits; whereas monoclonal antibodies have a unique specificity and are obtained *via in vivo* experiments in mice followed by *in vitro* phase in cell culture [185].

The detection of the Ab-Ag binding involves a secondary linkage between Ag and a detectable label (*e.g.*, enzyme, radiolabels, fluorophores, *etc.*). A competition takes place between the free Ag (analyte of interest) and a labeled Ag for a limited amount of specific Ab. According to the chemically linked label, immunoassays can be classified in radio-, enzyme, fluorescence, chemiluminescence, or bioluminescence immunoassay. A second categorization relies on the discrimination between homogeneous and heterogeneous competitive assays. In homogeneous immunoassays, the detection is directly performed after the mixing of the sample with the Abs. Heterogeneous immunoassays require a physical separation of the unbound analyte from the Ab-Ag complex prior to the detection [186,187].

Radioimmunoassay (RIA) was the first immunoassay developed in 1959. In RIA, the target antigen is radioactively labeled (^{14}C, ^{3}H, ^{125}I). The radioactive labeled Ag competes with the analyte of interest for the specific Ab. When Ab binds to the analyte, the free Ag remains in the supernatant and a radioactivity is measured. RIA has been applied to the sensitive analysis of many drugs but requires special training and instrumentation, as well as precautions due to the radioactive substances. This led to the development of the homogeneous enzyme multiple immunoassay technique (EMIT) where an enzyme (glucose-6-phosphate dehydrogenase) is attached to the Ag, and an enzyme substrate (NAD) is added to the mixture. In case of reaction, the substrate is transformed in NADH, a colored product. The increase in NADH absorbance is proportionally linked to the drug concentration. In absence of the targeted analyte of interest in the tested sample, which compete with Ag-enzyme for the limited amount of Ab, Ab binds to the Ag-enzyme conjugate, leading to a deactivation of the enzyme. Therefore, NADH is not produced and no colored signal is measured [188]. EMIT test is illustrated in **Fig. 1.6.A**. Another enzyme immunoassay is the enzyme-linked immunosorbent assay (ELISA), which varies in format but always involves an interaction of Abs covalently linked to an enzyme, with a reactant immobilized to a solid support [189]. Fluorescence-polarization immunoassay (FPIA) principle, highlighted in **Fig. 1.6.B**, relies on the competition of the analyte and a fluorescein-labelled Ag for the Ab under excitation with plane polarized light (λ = 485-490 nm). A small fluorescent molecule will rotate (at a relatively high rate compared to a large molecule, *e.g.*,

complex Ab-labeled Ag) during the adsorption of a photon and the emission of fluorescence, inducing a complete randomization of the plane of the emitted polarized light (depolarization). When the sample contains a high analyte concentration, the fluorescein-labelled Ag does not bind to the Ab, leading to a depolarization. In case of low analyte concentration, the labeled Ag binds to the Ab, thus forming a large complex which rotates at a low rate, exhibiting a high degree of polarization [190]. The kinetic interaction of microparticles in solution (KIMS) is a relatively recent assay where the Ag is bound to microparticles, as shown in **Fig. 1.6.C**. In the absence of targeted analyte in the sample, the microparticles become cross-linked by Ab due to the linkage with labeled Ag, producing large complexes that increase the turbidity of the solution. If a free Ag is present, these aggregates are not formed and the turbidity remains low. The latter is thus inversely proportional to the free Ag [188,191].

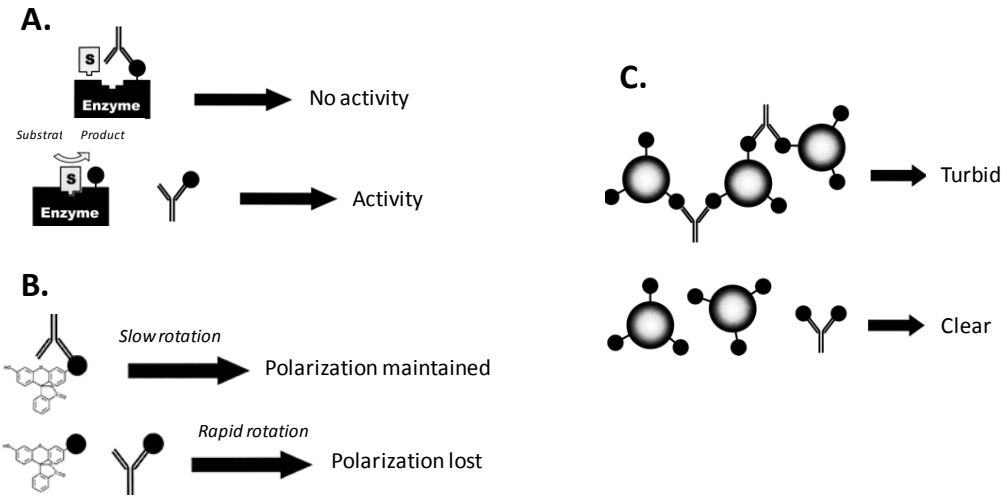

Figure 1.6. Illustration of the principle of EMIT (A.), FPIA (B.), and KIMS (C.) immunoassays. Adapted from [191].

Due to the additional costs and time involved by confirmation procedures, a high diagnostic specificity is required to avoid too many false-positive cases. However, immunoassays suffer from interferences from other exogenous xenobiotics or endogenous metabolites. Indeed, drug metabolites or compounds with similar structures may cross-react with the immunoassay when testing for a parent drug, leading to falsely elevated or, rarely, falsely lowered true concentration of the drug [192]. The qualitative results given by the immunoassay determination (positive/negative) rely on a specific calibrator concentration. If the drug concentration is higher than the calibrator cut-off, the result is positive; whereas a negative result expresses a drug concentration lower than the cut-off, which does not exclude its presence in the sample [193]. In case of cross-reactivity, an assay leads to a false-positive result which will be rejected by a more selective but time-consuming and cost-effective analysis. As an example, more than 30% of false-positive results, confirmed by GC-MS, were obtained with an amphetamine immunoassay (CEDIA Amphetamine, ThermoFisher Scientific) in a study carried out on almost 300 urine samples [194]. Cross-reactivity reactions were observed with numerous compounds, *e.g.*, bupropion, ephedrine and pseudo-ephedrine, oxycodone, MTD, ranitidine, or diphenhydramine. Potential cross-reactivity reactions are generally stated by the assays manufacturers. However, it has been shown that the cross-reactivity claims may not reflect the actual performance of the test due to batch-to-batch variability, lack of extensive validation of the test, or differences in the population [193].

Syva EMIT assays (Siemens Healthcare Diagnostics Inc.), Abbott TDx FPIA analyzer (Abbott Laboratories, retired in 2011), and Roche Online KIMS assay (Roche Diagnostics) belong to the most frequently used assays for drugs of abuse screening and have been evaluated in comparative studies [188,191,195,196]. The percentage of discrepancies in diagnostic sensitivity and selectivity is low (less than 10%) and depends on the screened compounds. The appropriate immunoassay should thus be selected according to the screened classes of compounds, the selectivity and sensitivity of the test, the cost of reagents and instrumentation, the speed and ease of the analysis, as well as the confirmation techniques available [195]. Regarding the screened compounds, an important drawback of all these assays is their lack of response to some designer drugs. Although mCPP has for example shown to cross-react with amphetamine in Roche Online KIMS assay [197], phenethlyamines such as 2C-B, 2C-Ts, 2C-I, and DOB were not detected with a commercial ELISA test, even at very high concentrations that exceeded those expected in forensic cases [198]. In such cases, state-of-the art chromatographic techniques are of fundamental importance for a reliable screening.

2.3.4 Chromatographic techniques

Immunoassays effectively present interesting features but are limited in diagnostic specificity and sensitivity. The emergence of highly selective and sensitive mass analyzers hyphenated to chromatographic techniques led to significant improvements in STA. GC-MS and LC-MS not only provide a reliable confirmation technique following an immunoassay determination, but can also be considered as state-of-the-art screening techniques due to their high selectivity and sensitivity, as well as their universal applicability leading to the detection of a wide range of xenobiotics and metabolites. When the identity of a xenobiotic is expected and limited to some classes of compounds, such as in WDT, a strategy consisting in an initial screening by immunoassay and confirmation by chromatography is generally sufficient (*targeted screening*), this strategy being possible only if the immunoassays are commercially available. For all other cases, a much extensive and high throughput screening is mandatory (*multi-target* or *general unknown screening*) where LC-MS and GC-MS take on particular importance [199]. Moreover, the time required for an analysis, long-standing considered limiting compared to immunoassays, tends to be dramatically reduced with high throughput techniques such as ultra-high pressure liquid chromatography (UHPLC) or fast GC. Hence, GC-MS and (UHP)LC-MS are today recommended in every discipline of forensic toxicology, as well as in clinical toxicology, even in case of acute intoxication.

2.3.4.1 Gas chromatography

To date, GC-MS remains the reference method for the confirmation of positive immunoassays and has been widely used in clinical and forensic toxicology since the first attempts in hyphenating GC with MS more than 50 years ago. GC-MS is also frequently used for volatile compounds determination and general unknown screenings [71,87,199].

GC involves the separation of volatile/semi-volatile and thermostable compounds according to their affinity for the stationary phase. A preliminary step of derivatization is mandatory for non-volatile and/or thermolabile compounds. The stationary phase generally takes place in capillary columns of 0.1 mm to 0.35 mm i.d., and of capillary length between 10 and 100 m. First capillary columns were

made of stainless steel, aluminum, gold, Teflon, or soft glass, but suffered from high metal oxide content and lack of flexibility [200]. Fused silica capillary columns have been used since the 1980s and their first commercial introduction. Bonded polysiloxanes stationary phases are widely used in toxicology and present a high thermal stability and good diffusivity. Multiple applications were developed with non-polar 100 % dimethylpolysiloxane (*e.g.*, HP-1, DB-1), slightly polar 5% diphenylmethyl-polysiloxane (*e.g.*, HP-5, DB-5), and 1 % vinyl 5 % phenylethyl-polysiloxane (SE-54) [201,202].

The most common ionization source to couple GC with MS is the electron impact ionization (EI) mode. In EI, the analytes diffuse in an electron beam produced by a heated filament and become ionized and fragmented under the $[M^{+\cdot}]$ form. In this *hard ionization* technique, a reproducible fragmentation pattern is obtained at typical electron energy of 70 eV, allowing for a comparison of the obtained fragments spectrum to reference libraries. In ion chemical ionization (CI), the ionization (*soft ionization*) is produced with a reagent gas, *e.g.*, methane or ammonia, leading to the formation of the molecular ion $[M+H]^+$ (positive ion CI) or $[M-H]^-$ (negative ion CI) which easily gives information on the molecular mass of the compound.

Confirmatory analyses are generally performed with GC-EI-MS in SIM mode, while full-scan mode is preferred for general unknown or multi-target screening [87,199]. For identification of compound with SIM experiments, some criteria are recommended by the reference guidelines [113]:

(i) The diagnostic ions should preferably include the molecular or precursor ion, and must be sufficiently characteristic of the compound's structure.

(ii) A minimum number of diagnostics ions are mandatory. For example, SOFT/AAFS guidelines encourage at least one qualifying ion in addition to a primary ion for each analyte and internal standard (IS), while WADA require at least three, and even more than three in the absence of chromatographic retention time data. If three diagnostics ions are not available, a second ionization technique shall be used (*e.g.*, CI) which should provide different diagnostic ions [71,203].

(iii) The relative abundances for the diagnostics ions must agree with permitted tolerance windows. Commonly used acceptance criteria by SOFT/AAFS for the ion ratios are ± 20% (relative) to that of the corresponding control. Some ion ratios being concentration-dependent, a comparison to a calibrator of similar concentration may be necessary [71]. The WADA expresses maximum tolerances windows in terms of absolute and relative differences. For example, if the relative abundance (% of base peak) of an ion is greater than 60%, the maximum tolerance window is ± 10% in absolute, *i.e.*, 50-70%. If the relative abundance is 25 to 50%, the maximum tolerance window is ± 20% in relative, *i.e.*, 32-48% for a relative abundance of 40%. The reasons for these absolute and relative tolerances remain unknown [203].

(iv) The retention times (RT) or retention index (obtained by comparing RT to reference *n*-alkanes) must be comparable to that of a reference, *e.g.*, RT should not differ by more than 2% or ± 0.1 min according to the WADA [203].

GC-MS in full-scan mode is the reference method due to huge available libraries of reference EI mass spectra that can be used for spectral comparison. This comparison leads to reliable identification,

even in the absence of reference substances in some specific cases. Suitable libraries should (i) take into account the artifacts produced during the sample preparation or the typical impurities or matrix components (ii) be built under standardized conditions, chromatographic conditions, and derivatization process, and (iii) contain spectra from xenobiotics and their metabolites [199]. The biggest mass spectral reference library is the Wiley Registry 10th Edition combined with NIST 2012 Mass Spectral Library that can be commercially purchased by Wiley (*ca.* € 8400.-). The library contains *ca.* 736,000 compounds and can be used in almost every formats proposed by current MS manufacturers. Another interesting library proposed by Wiley is the Mass Spectra of Designer Drugs 2013, by Peter Rösner, which contains more than 15,000 spectra covering the entire range of designer drugs until December 2012. The routine practice consists in a semi-automated search in the available libraries by the computer software after background subtraction. The quality of the match (*fit*) is expressed by a match factor generated for diagnostics ions (at least four) where 1.0 or 100% represents of perfect match. For a match considered positive, all of the diagnostic ions present in the reference spectrum must be present in the unknown sample (with exceptions in case of low overall abundance) [71]. It is worth mentioning that the final decision relies on the toxicologist's shoulders. Indeed, a full-scan reference spectrum for methamphetamine will for example show a match factor of ≥ 0.980 for multiple other compounds, *e.g.*, phentermine, propoxyphene, ephedrine, doxepin, or other phenethylamines [113].

Another computer tool was recently developed to aid the toxicologist in the identification which requires a high level of experience. The freeware program termed *Automated Mass Spectral Deconvolution and Identification System* (AMDIS) deconvolutes the spectrum and related information and matches it with a target library. AMDIS showed a better identification of low-abundant peaks and reduced the toxicologist's evaluation time by half [157,204]. The use of ADMIS programs is permitted by the WADA but shall be validated as part of the written procedure [203].

Even with this kind of sophisticated algorithms, the quality of the acquired spectra can be insufficient to perform a reliable identification, for example if the concentrations are too low for the GC-MS procedure. Tandem MS is an interesting alternative for general unknown screening due to its sensitivity. GC-MS/MS is generally used for quantitation purpose but has shown to be also applicable in full-scan mode to the screening of urine samples, extending the detection capabilities of GC-based procedures [157,205]. GC can also be combined with a TOF mass analyzer to improve the quality of the acquired spectra but its use is still limited in clinical and forensic toxicology [206].

Negative ion CI (NICI) can be considered to dramatically increase the sensitivity of compounds with electronegative moieties. Some BZD, *e.g.*, flunitrazepam and its metabolites norflunitrazepam, are poorly detected in blood samples by GC-EI-MS. Nevertheless, due to the presence of a halogen group, they can be detected using GC-NICI-MS. GC-NICI-MS may also be used for non-halogenated compounds, such as amphetamines, cannabinoids, or COC after a transformation to halogenated derivatives by agents such as trifluoroacetic anhydride, pentafluoropropionic anhydride, pentafluoro-1-propranolol, or boron trifluoride, which provide good GC properties while introducing an electronegative moiety [207-209]. Compared to EI, few or no fragment ions are produced with this soft ionization technique. Due to its selectivity for halogenated compounds and the lack of qualifiers fragments, very few applications of GC-NICI-MS for screening purpose have been proposed and only for targeted procedures, *e.g.*, for the determination of BZD in human hair [210].

The use of MS is recommended by the SOFT/AAFS guidelines for the confirmatory analysis. Indeed, a confirmation by other detectors such as nitrogen-phosphorous detector (NPD) or flame-ionization detector (FID) does not provide sufficient specificity, especially for medico-legal purposes [71]. Nevertheless, NPD, FID, electron capture detector, and surface ionization detection are still widely used combined with GC for targeted screening instead of immunoassays and occasionally for multi-target screening of basic/neutral substances. Headspace GC-FID remains a method of choice for the initial screening of simple volatile solvents such as ethanol, methanol, diethyl ether, or acetone in blood [211,212]. MS can also be coupled to these detectors, as shown with GC-NPD-MS [213] and GC-FID-MS [214] method developments. Nevertheless, the hyphenation of FID or NPD with high-vacuum MS is quite challenging. The recent development of atmospheric pressure ionization (API) sources for GC allowed overcoming the issues encountered with this coupling [215].

Although broadly used in toxicology, GC presents some limitations for the analysis of very polar or thermolabile compounds. Moreover, the conjugates cleavage and the derivatization step remain time-consuming despite the development of accelerated procedures. Finally, the carrier gas having no interaction with the compounds, the separation process solely relies on the analyte – stationary phase interaction, leading to a limited selectivity.

2.3.4.2 Liquid chromatography

LC involves the separation of compounds according to their affinity for both stationary and mobile phases. LC includes thin-layer chromatography (TLC) where the separation takes place on a planar glass or aluminum foil coated with the stationary phase, usually silica. TLC had its glory years in the 1960-1970s, whereas high performance TLC (HPTLC) took over in the 1980s, providing significant improvements in resolution and automation. Although losing ground to HPLC and above all UHPLC, it should be kept in mind that some clinical laboratories may not afford the expensive costs of GC or LC equipment and still use HPTLC today [4,216]. HPTLC also regained a modest interest with the development of automated HPTLC-MS equipment which provide multiple coupling options based on elution approaches (*e.g.*, surface sampling probe, forced-flow techniques) or desorption-based approaches (*e.g.*, laser light beam such as matrix-assisted laser desorption/ionization (MALDI), spray beam, ion bombardment) [217-220]. TLC and HPTLC being considered a *sub*-category of LC, a modification of the current terminology was even proposed by Morlock *et al.*, using *high performance column LC* (HPCLC) instead of *HPLC* [218].

In the 1980s, Bio-Rad Laboratories introduced the automated drug profiling REMEDi system based on HPLC with diode array detection (DAD) of λ = 193-305 nm. The analysis was carried out with four sequential columns, *i.e.*, two for the sample preparation and two for the analysis, and the system included a reference library of DAD spectra with more than thousand entries editable by the user [221]. REMEDi had a sample throughput of *ca.* 20-30 min and was widely used in many clinical laboratories for the screening of urine or blood without off-line preparation [191]. REMEDi showed its interest in the detection of tricyclic antidepressants, Z-drugs (zolpidem, zopiclone), or neuroleptics for which no immunoassay was available, but was rather limited for neutral or acidic compounds such as some BZD (nitrazepam, flunitrazepam) or barbiturates [222-225]. However, REMEDi system is not supported anymore since the end of 2008. The last years have thus seen many attempts in

replacing REMEDi system by LC-MS, LC-MS/MS, and LC hyphenated to high resolution MS (HRMS) [226-230].

First LC-MS systems were commercialized in the 1970s and waited until the 1990s to see the first toxicological applications appearing. Many improvements regarding ionization sources, hyphenation to hybrid or high resolution mass analyzers, or enhanced throughput with fast LC or UHPLC were carried out during the 2000s, leading to an important increase in its use. At the present time, LC-MS(/MS) is becoming fully competitive to the gold standard GC-MS for STA, not only providing complementary analysis for hydrophilic, thermolabile, and non-volatile analytes, but also for the analysis of a wide range of xenobiotics and their phase I and phase II metabolites with minimal sample preparation [132,160,231,232].

LC-MS hyphenation relies on API sources, including electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and atmospheric pressure photo-ionization (APPI) sources [160,231,233,234]. ESI is used in the larger majority of applications and is suitable for the ionization of highly polar to moderately non-polar compounds which present a broad mass range; while fewer applications were reported for APCI which is well suited for ionization of non-polar compounds and presents less matrix effects than ESI. However, APCI shows a limited polarity range, a higher background noise compared to ESI, and a relative incompatibility with thermolabile compounds. APPI shares the similar characteristics than APCI but has been rarely used in toxicology and only for quantitation purposes [235].

ESI process, illustrated in **Fig. 1.7**, relies on the application of a high voltage (± 2 – 5 kV) to a nebulizer needle containing the LC effluent, providing an electric field that penetrates into the liquid surface and leads to the formation of a meniscus at the ESI tip. A charge accumulation is observed, which causes a destabilization and a distortion of the meniscus to obtain the Taylor cone. When the solution reaches the Rayleigh limit, *i.e.*, the point at which coulombic repulsions on the surface charge are equal to the surface tension of the solution, droplets with excess of positive or negative charge detach from the tip and move towards the MS entrance. Ions are generated according to two models that are still debated: (i) the *charge residue model*, where the large droplets divide in smaller and smaller droplets, which eventually consist of single ions, or (ii) the *ion-evaporation model*, in which the increased charged density resulting from solvent evaporation causes a coulombic repulsion, resulting in a release of ions from droplets surfaces [233,236,237]. The desolvation is generally assisted by a gas (N_2) concentric to the effluent and a perpendicularly heated gas is frequently added for the same purpose. This soft-ionization process generally produces unfragmented protonated $[M+nH]^{n+}$ or deprotonated $[M-nH]^{n-}$ mono- or multicharged ions, as well as adduct ions with sodium, potassium, or other cations in positive mode.

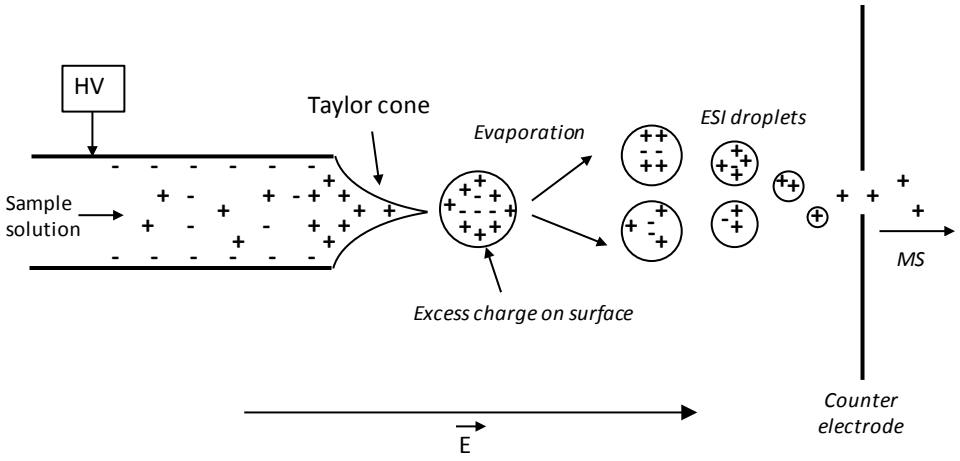

Figure 1.7. Schematic diagram of the electrospray process. Adapted from [238] and [237].

On the contrary of ESI which relies on a liquid-phase ionization, APCI ionization occurs in the gaseous phase, hence more adapted than ESI to hydrophobic analytes [160]. In APCI, the LC eluent flows through a heated nebulizer, pneumatically assisted or helped with a sonic device. The mixture of hot liquid and vapors expands into the atmospheric pressure interface, where it is ionized by a corona discharge, leading to a proton transfer from the solvent molecules to the analytes. Charge transfer produced positively $[M+H]^+$ or negatively $[M-H]^-$ monocharged ions [233]. APPI is based on a similar principle, but a vacuum-UV lamp is used instead of the corona and a dopant (*e.g.*, toluene) is generally added to the LC eluent to provide a source of easily photoionizable species and enhance the sensitivity [239]. The ranges of ionization for ESI, APCI, and APPI sources are highlighted in **Fig. 1.8**, according to the polarity and the molecular weight of the compounds of interest.

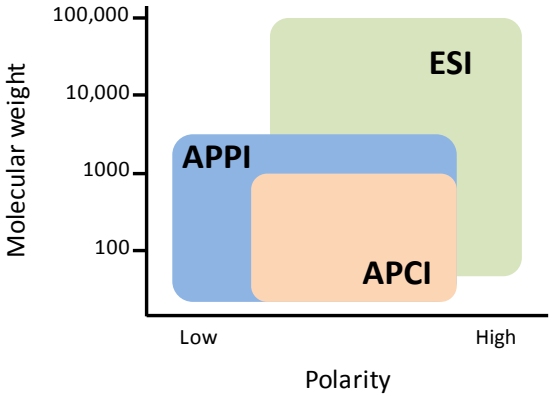

Figure 1.8. Range of ionization for ESI, APCI, and APPI as a function of molecular weight and polarity. Adapted from [240].

Over the two last decades, increasingly sophisticated and powerful LC-MS approaches were developed for STA in clinical and forensic toxicology. Early STA methods were based on single-stage LC-MS (using quadrupole) and LC-MS/MS instruments. Since API sources involve a soft ionization process, compounds fragmentation in single-stage LC-MS is performed with in-source collision-induced dissociation (CID). In-source CID consists in the acceleration of the ions by increasing the fragmentor voltage between the atmospheric pressure source and the high-vacuum of MS analyzer, making them collide with gas molecules and fragment [231,233]. Modern equipment allows for a fast switching between different fragmentor values during the run; compounds having different

fragmentation properties can thus be analyzed in one run [199]. Most single-stage LC-MS focus on multi-target screening procedures rather than general unknown screening.

LC-MS/MS experiments are generally performed with triple quadrupole (QqQ), ion trap (IT), or hybrid triple quadrupole-ion trap (QqQlinear IT) instruments. CID is performed in the high vacuum part of the MS, *i.e.*, in the second quadrupole q2 in QqQ and QqQlinear IT instruments, whereas fragmentation step and ion trapping step occur in the same quadrupole in IT systems, the latter also allowing to perform LC-MS^n experiments. For all MS/MS instruments, different acquisition modes can be selected for the equipment, depending on the operation mode of the quadrupoles, *i.e.*, *m/z* selection or scan, and are summarized in **Table 1.13**.

Table 1.13. Acquisition strategy for MS/MS experiments.

Acquisition mode	Q1	q2	Q3
Product ions scan (PR)	Selected *m/z*	CID	Scan
Precursor ions scan (PI)	Scan	CID	Selected *m/z*
Neutral loss scan (NL)	Scan	CID	Scan (with mass shift)
Selected reaction monitoring (SRM)	Selected *m/z*	CID	Selected *m/z*

SRM is mainly used for multi-target screening and confirmation purposes, as well as for quantitation. In SRM, a precursor ion(s) is/are selected in the first quadrupole Q1, fragmented in the collision cell q2, and the produced ion(s) is/are detected in the third quadrupole Q3. The required number of precursor and produced ions and their relative intensities for confirmatory purposes and identification are discussed in numerous guidelines. A consensus proposed by Rivier is based on the *identification points* (IPs). The total IPs obtained correspond to the sum of each piece of information from each part of the analytical step. For example, low-resolution MS/MS PI acquisition mode leads to an earned IP of 1.0 per ion, while it is equal to 2.0 per ion with high-resolution MS/MS PI mode [241]. Rivier suggests a minimum of IPs = 4, *e.g.*, one ion precursor and two product ions, or IPs = 5, *e.g.*, with two precursors ions, each with one product ion [242]. However, even with these criteria, the number of SRM transitions often remains insufficient, yielding a significant number of false-positive findings [243].

SRM may also be used for general unknown screening, as proposed and improved by several groups with a strategy based on PR mode and data- (or information-) dependent acquisition (DDA), schematized in **Fig. 1.9**. In DDA approach, MS/MS instruments can switch from a *survey* mode to a *dependent* (confirmatory) mode. An initial survey step is performed with a full-scan single stage MS mode. When at least one ion exceeds a preset threshold in MS mode, MS/MS is automatically and instantly triggered with product ion scan mode (PR). PR involves a selection of parent ion in Q1 prior to its fragmentation with a full-scan analysis of the fragments in Q3 (**Table 1.13**). The instrument is then switched back to the MS survey mode for the detection of new precursor ions. MS/MS spectra are matched to mass spectral libraries for identification. DDA avoid the preliminary selection of a precursor ion and is thus well adapted in general unknown screening approaches [132,160,231-233,243,244].

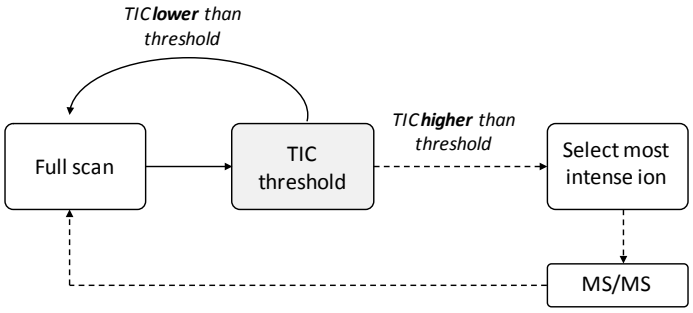

Figure 1.9. Representation of DDA strategy. The analyzer is set on full scan acquisition mode. When the total ion current (TIC) is higher than a fixed threshold, the most intense ion is selected and the acquisition switched to MS/MS mode to obtain the PR spectrum.

DDA has shown promising performance for multi-target screening with QqQlinear IT. The QqQlinear IT (QTRAP©, Applied Biosystems) combines the performance of a QqQ and an IT. In the QTRAP© instrument, the final quadrupole may be operating as a conventional RF/DC quadrupole or as a linear IT with axial ion ejection [245]. MS/MS experiments can be performed in enhanced product ion scan mode (EPR), where the precursor ion is selected in the first quadrupole Q1 and fragmented in q2, as for QqQ experiments. Fragments are then trapped in the last quadrupole Q3, leading to a high sensitive PR. As an example, a multi-target screening method applied to 301 compounds was developed with QqQlinear IT in EPR mode and the so-called *collision energy spread feature* which allows for the collection of data of three different collision energies in one single EPR spectrum, reducing the DDA cycle time and providing information for low- and high-mass fragments [246].

The main drawback with single stage LC-MS and LC-MS/MS approaches is the lack of mass spectral reference libraries. In GC-MS, huge libraries of EI mass spectra are available, as previously discussed. However, the fragmentation in LC-MS(/MS) presents a poor reproducibility between different instruments and can considerably vary, more on the product ions relative intensities than on the CID fragmentation pattern, and especially across different instrument brands. Therefore, most libraries are in-house built for each apparatus, which is tedious and leads to limited references [199]. Moreover, contamination and cleaning procedures of the ionization source can also have a significant effect in MS/MS spectra as shown by Gregov *et al.* [247]. The interpretation of unreferenced mass spectra is particularly challenging due to the limited number of fragments. Thus, it is recommended to combine proper and commercial libraries such as the *Wiley Registry of Tandem Mass Spectral Data, MSfor ID*, containing 10,000 spectra covering 1200 compounds [248], and use sophisticated search algorithms which weight relative or absolute fragment intensities to no or a minor extent, allowing for compensating variations between MS/MS spectra [249]. For example, SmileMS is new MS software based on the *X-Rank* algorithm, which first sorts the peak intensities of a spectrum followed by the correlation between two sorted spectra. A probability that a rank form an experimental spectrum matches a rank from a reference spectrum is then calculated and interpreted [228,250]

Another strategy to improve both general unknown screening and identification performance is the hyphenation of LC to HRMS, including TOF, hybrid quadrupole-TOF (QTOF), Orbitrap©, hybrid Orbitrap©-linear IT (LTQ-Orbitrap©), and Fourier-transform ion-cyclotron resonance (FT-ICR) mass analyzers. LC-HRMS instruments have been increasingly used over the last decade in forensic toxicology, especially for doping control, providing powerful information with accurate mass

determination and the corresponding empirical formula determination [206,251]. The resolving power R of a mass analyzer is its ability to separate ions with two different (*m/z*) mass-to-charge ratios and is measured according to **Eq. 1.1.**

$$R = \frac{m}{\Delta m} \quad \text{(Eq. 1.1)}$$

With Δm corresponding to the full width at half maximum (FWHM) of the height of the peak.

The mass accuracy is expressed with **Eq. 1.2** and depends on the peak shape, the ion abundance, the resolving power, and the external and internal calibration of the instrument.

$$mass\ accuracy\ [ppm] = \frac{(exact\ mass - measured\ mass)}{exact\ mass} \times 10^6 \quad \text{(Eq. 1.2)}$$

High resolution and high mass accuracy analyzers are generally defined with a resolution > 20,000 FWHM and a mass accuracy < 5 ppm. Modern TOF analyzers provide a resolution of 10,000-20,000 FWHM, even up to 50,000 with new instruments, and a mass accuracy lower than 2 ppm [206,252,253]. Another advantage of TOF/MS is the possibility of retrospective processing data without re-analysis, which can be very useful in case of new designer drugs or in doping control. TOF/MS principles and its characteristics are deeply discussed in **Chapter III**. The identification of xenobiotics relies on (i) the matching of the measured accurate mass and isotopic pattern to reference data, (ii) the comparison of retention time if a standard is available, and (iii) the interpretation of the metabolites pattern. A confirmation procedure is necessary as several xenobiotics and metabolites may share the same elemental formula and molecular mass [132,206,254]. In-source CID was proposed to overcome this limitation and obtain more structural information [230]. A similar approach is obtained using a QTOF instrument, where the third quadrupole Q3 of a QqQ system is replaced by a TOF/MS. QTOF instruments can work in several modes, *i.e.*, TOF mode only, PR or NL modes (**Table 1.13**), or fragmentation of all ions in the collision cell prior to TOF detection, with fixed or ramping collision energy [255-257]. Orbitrap© and LTQ-Orbitrap© (Thermo Fischer Scientific) provide excellent resolving power (> 100,000 FWHM) and mass accuracy (< 2 ppm), leading to powerful screening tools in combination with LC. LC-LTQ-Orbitrap© was already used in doping control for plasma screening with higher collision energy dissociation fragmentation [258]; whereas LC-Orbitrap© was used for urine screening with in-source CID [259]. However, their costs are still prohibitive for most of the toxicological laboratories. This is also the case for FT-ICR systems which present resolving power > 500,000 for low *m/z*. However, they are rarely hyphenated to fast separation techniques due to an acquisition speed limited to < 1 spectrum/s [253].

Besides HRMS technology, an increased interest has also emerged for ion mobility spectrometry (IMS) interfaced with MS (IMMS) which is one of the most rapidly growing areas of MS. IMS enables the differentiation of isobaric or isomeric ions on the basis of their charge-to-size ratios, providing valuable additional information to chromatographic and MS separations. The separation in IMS is based on the different ion mobilities in low or high electric field [260]. Four methods of interfacing IMS with MS have been described, *i.e.*, (i) drift-time ion mobility spectrometry, (ii) aspiration ion mobility spectrometry, (iii) differential-mobility spectrometry, and (iv) traveling-wave ion mobility

spectrometry [261]. All of these techniques provide different advantages and drawbacks in terms of resolution, duty cycle, and sensitivity. LC-IMMS (mainly with TOF/MS detection) systems have been also proposed and some applications can be found in the literature [260,262].

Apart from the developments in MS instruments, significant innovative supports and instrumentations for LC separation have emerged the past few years, leading to much faster analyses or higher separation resolution. Conventional reversed-phase LC (RPLC), which relies on the compounds partition between a volatile hydro-organic mobile phase and a silica-based with alkyl chains (C_{18}, C_8, C_4, phenyl, *etc.*) stationary phase, is widely used in bioanalysis but presents some limitations in terms of efficiency and analysis throughput. The following alternative strategies have been thus proposed:

(i) The use of columns packed with *sub*-2 µm particles, allowing for an increased linear velocity without loss in efficiency, due to the inverse proportion between particle size and both chromatographic efficiency and optimal phase velocity. Using *sub*-2 µm particles allows for (i) decreasing the column length with equivalent resolution, leading to a theoretical analysis time reduced by 9-times compared to conventional 5-µm packing or (ii) high chromatographic efficiency with longer columns. Since 2004, new UHPLC instruments have been commercialized that support the high pressure generated with *sub*-2 µm particles (up to 1300 bar). RPLC to UHPLC method transfer can be easily and directly performed with adaptation of injection and mobile phase flow rate. More than 100 columns packed with *sub*-2 µm particles are today commercially available [160,163,263-268].

(ii) High temperature LC (HTLC) provides faster separations without loss of efficiency, as well as a different selectivity than LC at ambient temperature [267,269]. HTLC may not be adapted to thermolabile compounds due to the heating of the mobile phase, usually at 90°C. Decreasing the mobile viscosity and the mass transfer resistance allows for an increase of the flow rate while keeping constant efficiency and acceptable pressure (< 600 bar), providing an interesting approach to increase the throughput with conventional columns and instruments. Both UHPLC and HTLC approaches can be combined in HT-UHPLC, leading to drastically decreased analysis times (as low as 1 min) without any loss of efficiency [263].

(iii) Increase in analysis throughput can be also obtained with monolith supports, which consist in a single rod of porous material (mesopores and macropores) possessing unique permeability and efficiency properties. A low pressure is generated during the analysis, allowing for high mobile flow rates. The second generation of silica monolith columns released in 2011 provides similar kinetic performance and lower backpressures compared to *sub*-2 µm particles [253,267,270]. However, few columns chemistries and geometries are commercially available yet and monolith supports do not resist to high temperature or relatively extreme pH values.

(iv) Columns with *sub*-3 µm core-shell particles (*Fused Core* technology) appeared in 2007 and present similar performance than UHPLC with a 2 to 3-times lower generated backpressure. Columns are packed with 2.6-2.7 µm superficially porous particles which consist of a solid inner core of 1.7 to 1.9 µm diameter and a porous outer core of 0.35-

0.5 µm. Numerous columns geometries and chemistries with *sub*-3 µm core-shell particles are currently available, which make this core-shell technology really competitive for fast LC analysis with conventional HPLC instruments [253,266,267,270].

(v) Hydrophilic interaction liquid chromatography (HILIC) involves the use of polar stationary phases with a hydro-organic mobile phase typically composed of > 70% of organic solvent (acetonitrile). The retention mechanism is not well understood yet but mostly consists of partitioning between the bulk mobile phase and a layer of mobile phase enriched with water that is partially immobilized on the stationary phase surface, as well as adsorption, dipole-dipole, and ionic retention contributions. HILIC is well suited for the analysis of polar compounds and presents an increased MS sensitivity due to the higher amount of organic solvent. An alternative selectivity is observed compared to conventional RPLC, providing an additional and orthogonal chromatographic mode for screening or confirmation purposes [160,271-274].

UHPLC is today increasingly used combined with HRMS. It is worth mentioning that the inherent constraints of this coupling should be considered with attention when developing new methods, such as the use of mobile phase flow rates compatible with ESI-MS process, the minimization of extra-column band broadening, and the selection of an adapted data acquisition rate compatible with the narrow peak generated in UHPLC [253]. Thereby, UHPLC-HRMS provides a very powerful and reliable tool for general unknown screening which strongly decreases the analysis time while enabling reliable compounds identification.

2.3.5 Current and future trends in systematic toxicological analysis

GC and LC-based analytical techniques are involved in the large majority of comprehensive STA strategies. Both techniques have presented significant innovative developments over the last decades, leading to powerful screening tools that have been herein exposed. However, despite these considerable improvements, none of these techniques can be used as a single approach. Two techniques or more are still required to reach an exhaustive outlook of the sample composition, due to the matrix complexity, the number of potential xenobiotics and metabolites, their large differences in physico-chemical properties and concentrations, as well as instrumental or technical limitations. The following years are expected to provide effective answers to these complex analytical problems.

Besides LC and GC, other techniques such as capillary electrophoresis (CE) or supercritical fluid chromatography (SFC), both currently seen as *Davids* in the world of *Goliaths*, shall also be considered as alternative or complementary analytical tools. Following Chapters will aim at showing the high potential of CE-MS in clinical and forensic toxicology.

SFC has a history that is as long as that of HPLC, since its first mention dates back to the 1960s. In SFC, the mobile phase consists of a supercritical fluid which represents the physico-chemical state of a substance occurring when temperature and pressure are above the critical point. This single phase presents unique characteristics, *i.e.*, (i) a density similar to a liquid solvent, providing a good solvating power, (ii) a viscosity and diffusivity similar to the gas state, inducing a lower pressure within the column and a faster analysis [275-277]. With a critical temperature T_c = 31 °C and a critical pressure

P_c = 73 atm, carbon dioxide is generally used as the mobile phase in combination with methanol or other solvents and additives to tune the selectivity and to allow for the analysis of polar compounds. Rather used for preparative chromatography or chiral separations, SFC had for years not found a wide acceptance in toxicology. Nevertheless, since few years SFC is regaining inquisitiveness thanks to numerous recent developments and concerns. The extreme shortage of acetonitrile in 2008 and 2009 rose new challenges in LC due to its wide use in every step of the analytical process, bringing attention to alternative techniques such as SFC which did not depend to the supplying. Moreover, the advent of the *green chemistry* concept in the 1990s promoted the use of non-toxic or less solvents for health and environment protection. Recent hyphenation of SFC to MS allowed for its applicability to the detection and quantitation of low concentrations, as encountered in biological matrices. Finally, the most promising improvement on the basis of UHPLC is the emergence of ultra-high performance SFC (UHPSFC) technology with the commercialization of new instruments able to work with columns packed with *sub*-2 µm particles, providing excellent kinetic performance. Various stationary phase chemistries are already available (*e.g.*, bare silica, hybrid silica, 2-ethylpyridine). UHPSFC technology, especially in combination with MS detection, will probably be more extensively used in bioanalysis for high-throughput or high-resolution analyses [278].

2.4 Quantitative procedure

After compounds confirmation and identification, the second step of a comprehensive toxicological process is the quantitation, generally required by the reference guidelines in forensic toxicology. In emergency departments, quantitation is essential for a meaningful interpretation and treatment of poisonings, especially if reference data and decision criteria (nomograms) are available or to assess the prognosis [87]. Moreover, quantitation of serum or plasma concentrations of medications is required in TDM for dose optimization.

2.4.1 Sample pretreatment

2.4.1.1 Biosamples

Plasma and serum are clearly the samples of choice for compounds quantitation because they show the best correlation with pharmacologic effects when the distribution equilibrium with other tissues has been reached, except for psychoactive drugs presenting tolerance or dependence properties. Quantitation in plasma or serum samples is required in numerous fields, particularly for those involving court proceedings. Oral fluid can also be considered in addition or as a surrogate to plasma and serum (*e.g.*, for DUID purpose), due to the major and readily presence of parent drugs as well as the similar pharmacokinetics properties than plasma/serum. Indeed, there is a known relationship between plasma and oral fluid concentrations which can be estimated depending on the pH of the oral fluid, the protein binding affinity of the drug, and its pKa values. Other factors having an influence on the concentration are the volumes of oral fluid and diluents used in commercial kit for sample collection, which are both often unknown, and the potential residual amounts of ingested, smoked, or snorted xenobiotics in the mouth cavity. Even if the pharmacokinetics of drugs have shown to be more complex in oral fluid than in blood, drugs quantification in oral fluid remains interesting in WDT, TDM, or DUID [142,143].

Urine is not the primary choice for compounds quantitation due to the very little correlation existing between urine and blood xenobiotics concentrations, as well as urine concentrations and pharmacological effects. Nevertheless, quantitation of xenobiotics and/or phase I and phase II metabolites is frequently carried out, as for example in doping control. In urine samples, the concentration is affected by numerous factors, *i.e.*, the fluid intake, the time of urine collection (24-hour sampling *versus* spot collection), the pH, the intra- and interindividual metabolism variability, the glomerular filtration rate, and the perspiration [128,279]. The measured concentration can be corrected with specific gravity (d) and/or creatinine values (*normalization* procedure). The WADA requires a correction for endogenous steroids quantitation with a reported concentration value adjusted for the specific gravity of the urine, according to **Eq. 1.3**, based on the Levine-Fahy equation [279-281] and a reference d value of 1.020.

$$Concentration_{1.020\ ng/mL} = \frac{(1.020 - 1)}{(d_{sample} - 1)} \times Concentration_{measured\ ng/mL} \quad \text{(Eq. 1.3)}$$

Based on a similar approach, correction of the concentrations with measured creatinine was also proposed with **Eq. 1.4**, d and creatinine showing a high correlation for both spot samples and 24-hour sample collection. Reference value of creatinine was fixed at 100 mg/dL [281].

$$Concentration_{100\ mg/dL} = \frac{100}{Creatinine_{sample}} \times Concentration_{measured\ ng/mL} \quad \text{(Eq. 1.4)}$$

This creatinine-based normalization was also found acceptable especially for highly diluted urine samples. Some limitations are encountered with both normalization approaches in case of extreme dilution/concentration of urine, and differences can be observed in certain disease states.

The quantitation of xenobiotics and metabolites in hair provides some information regarding the consumption, even if there is no inter-individual correlation between the dose or frequency of consumption and hair concentration. Retrospective studies allow for an interpretation of hair concentration for some drugs of abuse including COC, heroin, or MDMA, with a comparison of the measured concentrations to those found in usual consumers, although the data provided by usual consumers do not inform about their consumption habits. Another strategy is to apply a statistical evaluation of the measured concentration to help in the interpretation [146].

An interesting application of hair quantitation is the determination of ethyl glucuronide (EtG) in chronic alcohol monitoring. Indeed, EtG concentration in rat hair has demonstrated to reflect the EtG concentration in blood, whereas an increased ethanol administration led to a proportional increased EtG concentration in hair [282]. In human, the analysis of EtG in a hair proximal segment (0 - 3 cm) can be interpreted according to fixed cut-offs based on the concentration of EtG and the limit of quantitation (LOQ) of the method [283]:

(i) 0 < EtG concentration ≤ LOQ: *absence* (but does not exclude a low risk of alcohol consumption);
(ii) LOQ < EtG concentration ≤ 7 pg/mg: *low risk consumption* (exclusion of abstinence);
(iii) 7 < EtG concentration ≤ 30 pg/mg: *at-risk consumption*; and
(iv) > 30 pg/mg: *heavy consumption*.

EtG incorporation in hair is not influenced by gender, age, or body mass index and its quantitation is thus useful and very promising in alcohol monitoring.

2.4.1.2 Sample pretreatment

Sample preparation has been for a long time considered as a primary source of analytical errors that affect the accuracy of the quantitation, especially with off-line procedures. Automation or semi-automation of PP, SPE, or LLE/SLE procedures, for example with semi-automated 96-well plates or column switching, led to significant improvements in quantitative performance [284,285].

Using GC or LC combined with rather poor selective detectors (*e.g.*, UV-DAD, FID, *etc.*) generally requires an upstream selective sample preparation for target extraction of the compound(s) of interest in blood or urine samples. In these cases, SPE is often selected due to the great variety of sorbents commercially available. Today, there is an increase in GC-MS and LC-MS(/MS) instruments used in daily practice and the high selectivity provided by MS detection allows for more simple and rapid clean-up procedures. Moreover, consideration about costs, analysis throughput, and organic solvents consumption tends to replace SPE and LLE by easier approaches, such as DBS or sample dilution. This is particularly true when chromatographic techniques are coupled to HRMS.

Quantitation of non-volatile or polar analytes by GC-based techniques requires a compounds derivatization after extraction. An enzymatic or acidic hydrolysis of phase II conjugates is also mandatory in specific cases, as for example in doping control for the quantitation of testosterone and epitestosterone [280].

An important issue encountered with non-selective sample pretreatments when using LC-MS(/MS) is the potential effects of the matrix interferents on the ionization which can significantly affect the accuracy of the quantitation, leading to a wrong interpretation of the results. With a simple urine dilution or plasma PP, the sample clean-up is not effective and numerous matrix components such as residual proteins, (phospho)lipids, sugars, salts, other endogenous or exogenous compounds (*e.g.*, anticoagulants) as well as mobile phase constituents (*e.g.*, ion pairing agents) are also injected. When co-eluting with the targeted compounds, these interferents can lead to signal suppression or enhancement during the ionization process. Ion suppression or enhancement results from a change in the efficiency of droplet formation or evaporation due to the presence of non-volatile or less volatile solutes which affect the amount of charged ions in the gaseous phase. ESI is more prone to matrix effects than APCI and APPI, and more ion suppression is observed with positive than negative ionization mode. Compounds with higher mass have a predisposition of suppressing the signal of smaller molecules, whereas more polar analytes are more susceptible to suppression. These so-called *matrix effects* are particularly important for complex matrices such as blood or post-mortem samples if the clean-up is not optimal and are problematic in both STA (potential compounds overlook) and quantitative (inaccurate concentration measurements and lower sensitivity) procedures [132,160,165,286-288].

The following strategies can be implemented to overcome matrix effects [286,289,290]:

(i) Use a more selective sample preparation instead of non-selective procedures to have a more efficient clean-up. Mixed-mode SPE sorbents with ion exchange interaction provide a better clean-up than sorbents based on reversed-phase mechanism [291]. SPE can lead to matrix effects due to the procedure itself, which is not the case with LLE who presents less ion suppression [292].

(ii) Substitute trifluoroacetic acid (TFA), an ion-pairing agent leading to strong ion suppression, by weaker and volatile acids such as acetic or formic acids. The majority of matrix effects occurring in the solvent front of the run in RPLC, the chromatographic conditions (*e.g.*, column packings, elution gradient) can be modified to change the elution of the targeted compounds in a region where ion suppression is not observed [286]. Ionization variations have also been observed when using methanol of different qualities and providers in the mobile phase, probably explained by the presence of contaminants in the solvent [289].

(iii) Opt for APCI or APPI sources instead of ESI. However, this solution is rarely selected due to the lower sensitivity observed for numerous compounds with both sources. ESI source geometry can also lead to differences in the matrix effects. For example, the signal suppression was found higher with Z-spray ion sources than orthogonal spray configuration in a study [293].

(iv) Build the calibration curve during both validation procedure and routine quantitation with calibration standards prepared by spiking compounds in reconstituted or blank matrix.

(v) Use a suitable internal standard (IS) during the quantitation. An internal standard should have chemical and physical properties as similar to the analyte as possible and should be added to the sample at the earliest possible stage in the procedure to compensate for extraction or ionization variability. Isotopically-labeled IS are supposed to show the same extraction and ionization behavior and are thus strongly recommended, especially the deuterated analogues of the analyte with at least three deuterium atoms. Ideally, one deuterated IS should be used for each analyte quantified whenever possible. When no deuterated standard is available or for cost-effective reasons, a representative deuterated IS can be used for more than one analyte. Analog compounds are not recommended due to their potential presence in the sample but might be useful in some instance, in this case drugs analogs which are not licensed should be considered. The added concentration of the IS should be representative of the case under investigation. In MS/MS analysis, the selection of fragments should allow to retain the labeled atoms when possible. It is also important to evaluate if the IS suppresses or enhances the analyte signal and *vice versa*. In case of modification of analyte ionization efficiency by the IS, incorrect concentrations will be calculated depending on the level of calibrator compared to the sample. Indeed, low-concentrated calibrator will be more prone to ion suppression/enhancement than high-concentrated calibrator. Therefore, only IS which do not show any suppression or enhancement should be selected [71,87,160,286,294-296].

The evaluation of matrix effects is an integral part of the validation procedure of any LC-MS based method and is discussed in **Section 2.4.3.2.1**.

2.4.2 Chromatographic techniques

GC-MS and LC-MS in SIM mode as well as LC-MS/MS in SRM mode are recognized as the gold standards quantitative methods. Besides single-analyte quantitation procedures, where only one targeted compound and its respective metabolite(s) are quantified in a run, multi-analytes strategies have been developed to allow for a simultaneous quantitation of numerous analytes and their respective metabolites within the same run [199].

With the advent of UHPLC technology, UHPLC-MS/MS with QqQ is increasingly used for compounds quantitation and generally provides at least a four-order linear range. QqQ shows the advantage to perform rapid SRM experiments which are mandatory due to the sharp chromatographic peaks obtained with UHPLC [253]. A number of 12-15 points per peak are required for accurate quantitation. Modifications of separation conditions (*e.g.*, mobile phase flow rate, column length) or use of time segments SRM experiments shall be envisaged to ensure a sufficient number of points. If only one SRM transition is used for compound quantitation, interference from isobaric compounds sharing the same transition can occur, leading to inaccurate quantitation. It is therefore recommended to use a second SRM transition or to ensure a sufficient selectivity with the sample preparation and the LC conditions in case of compounds presenting only one major fragment [160]. The use of LC-HRMS can also be considered for compound quantitation. It has been recently shown with a large set of low-molecular weight compounds that an accurate quantitation can be performed with HRMS having a resolving power of ≥ 20,000 FWHM, provided that the mass extraction window used for the post-acquisition data processing is carefully selected. Using LC-HRMS systems for quantitative purposes presents another advantage regarding the possibility of subsequent data treatment and information about other compounds present in the sample [297].

2.4.3 Bioanalytical method validation

The validation of analytical methods prior to their use in routine is required in every quality assurance program. In forensic toxicology, it is sometimes accepted in specific cases that a full validation for each method offered by a laboratory cannot be conducted due to the wide range of analytes or their infrequency; these methods should therefore demonstrate their “fitness for purpose” and validated if they become routine procedures [71,75]. The validation of methods is required by the ISO/IEC 17025:2005 standard to provide objective evidence that the method fulfill the requirements for a specific intended use [36]. Numerous guidelines and recommendations have been proposed for method validation in bioanalysis.

2.4.3.1 Guidelines

The most relevant recommendations are provided in the *Guidance for Industry: Bioanalytical Method Validation* of the Food and Drug administration (FDA) [51,298], the *Guideline on bioanalytical method validation* of the European Medicines Agency (EMA) [299], the *Standard Practices for Method Validation in Forensic Toxicology* of the SWGTOX [73], the *Requirements for the validation of analytical methods* of the GTFCh [53], and the *Forensic Laboratory Guidelines* of the SOFT/AAFS [71]. The relevant validation criteria are presented hereafter. In order to help the analyst in the systematic

validation of a new method, some validation protocols are also provided by the Société Française des Sciences et Techniques Pharmaceutiques (SFSTP) [300-302], the International Conference of Harmonization (ICH) [303], and in a comprehensive paper of Peters *et al*. [304].

2.4.3.2 Validation criteria

2.4.3.2.1 Selectivity

The ability of an analytical method to unequivocally differentiate and quantify the compound in the presence of other components in the sample is termed *selectivity*. At least six different sources of blank matrix should be used to test the selectivity. It has been also proposed to evaluate more than ten to twenty sources of blank samples due to the high probability of relatively rare interferences encountered when only analyzing six blank specimens. The selectivity can be evaluated by proving the lack of response in each blank matrix at the lower limit of quantitation (LLOQ), with no signal interfering with the targeted compound and its respective IS.

Matrix effects can be evaluated following two approaches, *i.e.*, qualitative and quantitative.

The qualitative evaluation relies on a post-column infusion system which was first proposed by Bonfiglio *et al.* in 1999 [305]. In this approach, an analyte solution is continuously infused into the eluent of the LC column *via* a post-column tee connection and the prepared blank matrix is injected. In case of ion suppression or enhancement, a decreased or increased detector response is observed, respectively. This evaluation is particularly useful to detect potential matrix effects and to determine when they occur in the separation.

The quantitative approach was proposed by Matuszewski *et al*. and involves the analysis of two samples, *i.e.*, (i) a neat spiking solution in water (*standard*, A) and (ii) the blank matrix spiked with the targeted analyte after sample pre-treatment (B). Matrix effect is calculated with the ratio between the peak areas in B and in A, and is usually expressed in percent. A result of 90 % expresses a 10-% ion suppression, while a result of 110 % represents a 10-% ion enhancement [306]. An alternative estimation of matrix effects can be done according to **Eq. 1.5**:

$$Matrix\ effects = \left(\frac{B}{A} - 1\right) \times 100\% \qquad \text{(Eq. 1.5)}$$

Where negative results express an ion suppression while positive results indicate an ion enhancement [288].

In case of matrix effects, the quantitation (with systematic IS correction) is still acceptable as long as the sensitivity is sufficient and the repeatability of matrix effects between matrix batches is ≤ 15%. Matrix effects should not be higher than ± 25% for average value of blank matrix samples [53,73]. It is worth mentioning that the qualification or quantitation of matrix effects is also recommended in STA procedures. However, in this context, due to the large number of screened analytes, the evaluation is generally performed on a representative set of compounds [288].

2.4.3.2.2 Calibration and response function

A *calibration curve* represent the relationship between the known concentration of calibration standards and the corresponding response (*e.g.*, the ratio between peak areas of analyte and IS). Calibration standards should be prepared in the same biological matrix as the samples by spiking it with known concentrations. A sufficient number of calibrators are required to ensure an accurate quantitation and must cover the whole anticipated range of analytes concentrations (*working range*). Generally five to eight non-zero calibrators are required, including the LLOQ, with two to six replicates *per* level. The simplest regression model such as linear regression is recommended to describe the concentration-response relationship. Generally, due to the heteroscedasticity (random variances) of the data covering three or four order of magnitude, ordinary least square (OLS) regression model is not adapted and weighted OLS (*e.g.,* $1/x$, $1/x^2$), square root, log transformation, or non-linear regression models are mandatory [304]. The quality of the calibration curve is typically evaluated with the correlation coefficient. Recent or updated guidelines recommend to visually evaluate the calibration model with standardized residual plots which allow for checking for outliers and determining if the model adequately fits to the data [73]. For routine analysis, fewer calibration samples (levels and replicates) can be used when a linear calibration model is used.

The LLOQ is the lowest concentration that can be quantitatively determined with a defined accuracy (trueness and precision) within the acceptance limits. The acceptance limits are generally fixed at ± 20 % for the precision and ± 20 % for the bias. The LLOQ serves as the lowest concentration of the calibration curve, and the highest concentration is defined as the *upper limit of quantitation*.

The *limit of detection* (LOD) represents the lowest concentration at which the analyte can be differentiated from background noise. Numerous approaches can be implemented to estimate the LOD of an analytical procedure. In most cases, the LOD is determined in at least three sources of blank matrix samples spiked with decreased concentrations, analyzed three times at each concentration, and defined at the concentration where the signal-to-noise ratio is ≥ 3. LOD can also be estimated based on the standard deviation (SD) of the response and the slope of the calibration curve.

2.4.3.2.3 Accuracy

The *accuracy* is the expression of the total error of the analytical method, *i.e.*, systematic (*bias*) and random (*precision*, estimated by variances, SD, or relative standard deviation, RSD) errors. Relative bias is used to express the *trueness* of an analytical procedure which represents the closeness of agreement between the mean value obtained from a series of measurements and the accepted reference value (estimation of true unknown value) [301,307]. *Accuracy* is often incorrectly used to describe the trueness only. The *precision* represents the closeness of individual measures obtained from multiple aliquots of a single homogeneous volume of biological matrix, and encompasses repeatability, intermediate precision, and reproducibility [303]. *Repeatability*, also termed *within-day*, *intra-batch*, or *within-run precision*, expresses the precision obtained within a short interval of time (intra-series or intra-day). *Intermediate precision*, also referred to as *inter-batch* or *between-run precision*, represents the within-laboratory precision, *i.e.*, different days, operators, or equipment. Both repeatability and intermediate precision should be estimated during the validation procedure.

Reproducibility is the precision observed between laboratories and is generally not determined, except if a method has to be used in different laboratories.

Relative bias for trueness and variances for repeatability (s_r^2) and intermediate precision (s_R^2) expressed by RSDs are estimated with analysis of quality control or validation standard samples at low, middle, and high concentration levels. Acceptance criteria are ± 15 % relative bias for trueness and ± 15 % RSD for precision, except for LLOQ. The total error of a procedure, including random and systematic errors, can be represented with accuracy profiles. An example of accuracy profiles is shown in **Chapter III** for the validation of COC and MTD in urine samples by CE-MS. Relative bias (δ), upper and lower tolerance or confidence limits, and acceptance limits (λ) are represented in the profile. An analytical procedure is validated if the requirement given by the **Eq. 1.6** is fulfilled.

$$P(|\delta| < \lambda) \geq \beta \qquad \text{(Eq. 1.6)}$$

With β being the probability that a future determination falls within the acceptance limits (generally 95%) [307]. Acceptance limits are generally fixed at ± 30 % for bioanalytical methods.

2.4.3.2.4 Uncertainty

The estimation of uncertainty is recommended by ISO/IEC 17025:2005 standard. Uncertainty is a parameter (*e.g.*, SD) associated with the results of measurement that characterizes the dispersion of the values that could reasonably be attributed to the measurand, *i.e.*, a particular quantity subject to measurement [308,309]. Uncertainty is practically defined as a probability or level of confidence. Indeed, a quantitative result takes the form of $a \pm ku$ where u is the standard uncertainty (*e.g.*, SD) and k a coverage factor. Expanded uncertainty U is an interval in which the result of a measurement resides with a defined probability. When x = 2, the result $a \pm U$ represents a 95% level of confidence where the true value would be found. Individual component uncertainties u_i (resulting from sampling, sample preparation, *etc.*) are taken into account to express the overall U. A guide has been edited by the Joint Committee for guides in Metrology and provides the required information to evaluate the uncertainty of measurement data [309].

2.4.3.2.5 Stability

Stability should be evaluated to ensure that each stage of the analytical procedure, including sample storage and sample processing, does not affect the concentration of the analyte. It depends on the analyte, the matrix, the container system, and the storage conditions. Recommended are the estimations of freeze/thaw stability (three freeze/thaw cycles), short-term stability (4-hour bench top at room temperature), and long-term stability (refrigerated or frozen at the storage temperature). Stability of prepared samples should also be evaluated (*post-preparative stability*), including the time spent in the autosampler prior to the injection.

2.4.3.2.6 Robustness/Ruggedness

The *robustness* or *ruggedness* is defined as the capacity of the analytical procedure to remain unaffected by small but deliberate variations in method parameters (*e.g.,* mobile phase composition, temperature) [303,310]. It provides an indication of the reliability of the method in the routine use. Robustness should be evaluated in the early development of an analytical method. Methodologies

based on design of experiments can be used for robustness evaluation with implementation of Plackett-Burmann or fractional factorial design of experiments after selection of operating and environmental factors. Another approach is the use of Quality by Design and the related Design Spaces approaches. The Design Space is a *sub*-space where the assurance of quality has been proved and is a measure of the robustness of a procedure. The determination of the Design Space of a procedure leads to valuable information on how often the requirements will be met to provide reliable data and assurance of the quality [311].

2.5 Toxicological data interpretation

Numerous considerations have to be taken into account for the toxicological interpretation of the results. Relevant factors having an impact on the data interpretation are the specifications of the analytical procedure, the type of specimens and collection, the initial dose, the frequency and duration of consumption, the route of administration, the date of collection, the possible tolerance/addiction, the pharmacokinetic properties, the drug-drug interactions, the circadian cycle, the physical activity, the pregnancy, the disease status, or genetic factors such as age, gender, and polymorphisms (inter-individual phenotype differences). In case of post-mortem analysis, some additional factors have to be considered, *e.g.*, post-mortem redistribution, sample integrity, and analyte stability.

Interpretation thrives on a mix of logical and intuitive decision. The last decade saw an emergence of a new paradigm based on probabilistic approaches, with the use of likelihood ratios and Bayesian reasoning. This probabilistic approach is already known in clinical toxicology and increasingly considered in forensic fields [312-315]. It is hereafter briefly presented with clinical examples.

The likelihood ratio (LR) expresses the probability of obtaining the evidence E given H_0 *versus* alternative H_1 hypotheses according to **Eq. 1.7**:

$$LR = \frac{p(E|H_0)}{p(E|H_1)} \quad \text{(Eq. 1.7)}$$

In clinical chemistry, $p(E|H_0)$ represents the probability of the result in the affected population and $p(E|H_1)$ the probability of the result in the normal population. The higher the LR value, the higher the probability of the patient to belong to the affected population. The odds form of Baye's theorem is then expressed by **Eq. 1.8**:

$$\frac{p(H_0|E)}{p(H_1|E)} = \frac{p(E|H_0)}{p(E|H_1)} \times \frac{p(H_0)}{p(H_1)} \quad \text{(Eq. 1.8)}$$

$$Posterior\ odds = LR \times prior\ odds$$

Where the *prior odds* are the odds of both hypotheses before the scientific evidence; it is represented by the *prevalence* in clinical chemistry, *i.e.*, the frequency of the affection in a *sub*-category of the population. Both *prior odds* and LR knowledge is thus necessary for results interpretation. This kind of probabilistic tools will likely be increasingly used in the future taking into consideration the data interpretation with holistic approaches.

3 Contribution of CE-MS in clinical and forensic toxicology

3.1 Capillary electrophoresis

Electrophoresis, which is defined as the movement of charged compounds under the influence of an electric field, has been first described by Tiselius in 1937 who observed the migration of proteins in a buffer in different directions and at different velocities, depending on their charge and mass. Capillary format was first used by Hjerten who presented in 1967 the use of 1- to 3-mm i.d. quartz capillaries. The early 1980s saw the emergence of 200-µm i.d. capillaries in glass and Teflon, followed by 75-µm i.d. capillaries with the works of Mikkers, Everaerts, Jorgensen and Luckacs [316]. Due to the greater area-to-volume ratio encountered with a capillary format, efficient heat dissipation was obtained, leading to much better efficiencies. Indeed, the Joule heating effect, *i.e.*, the heat produced when an electric current flows through a conductor, leads to temperature gradient in the capillary, inducing dispersion, peak broadening, and lower resolution [317].

The simplest CE mode of operation is capillary zone electrophoresis (CZE), in which a narrow-bore fused-silica capillary (*ca.* 30 to 100 cm of total length, 25 to 150 µm i.d., and externally coated with polyimide) is filled with an aqueous background electrolyte (BGE) at a defined pH. A small plug of sample is injected into the capillary by positive or negative pressure (*hydrodynamic* injection, HD), or by electrokinetic pumping (*electrokinetic* injection, EK), then an electric field is applied. Capillary tips are immersed in vials containing the same BGE as well as electrodes connected to the high voltage power supply (≤ 30 kV). The separation relies on the differences in the analyte velocity v (cm/s) in an applied electric field E (V/cm), as expressed by **Eq. 1.9**:

$$v = \mu_{eff} E = \mu_{eff} \frac{U}{L} \quad \text{(Eq. 1.9)}$$

Where μ_{eff} is the effective mobility of the analyte (cm^2/Vs), U the applied voltage (V), and L the capillary length (cm). The effective mobility is constant for a given ion and conditions and depends on its net charge q, its radius r, and the viscosity η of the BGE, as expressed by **Eq. 1.10**:

$$\mu_{eff} = \frac{q}{6\pi\eta r} \quad \text{(Eq. 1.10)}$$

According to **Eq. 1.10**, the separation of charged compounds is proportional to their charge-to-size ratio, where small and highly charged species will have a higher effective mobility than large and single-charged compounds. Another transport mechanism involved in the capillary is the electro-osmotic flow (EOF) which is observed in case of charged capillary wall. Indeed, at *ca.* pH ≥ 4, the silanol groups of the silica capillary are mostly deprotonated and the surface of the capillary wall is negatively charged, inducing the shifting of the counterions present in the BGE towards the silanol groups due to electrostatic forces. This mechanism leads to the formation of a first layer of counterions termed *fixed layer* or *Stern layer*. A second cations-rich layer, the *diffuse layer*, is also formed next to the Stern layer due to the remaining negative charges on the capillary wall. This double layer formed by Stern and diffuse layers creates an electric potential difference very close to the capillary wall called the *zeta potential* ζ. When applying the voltage, the cations forming the

diffuse layer move towards the cathode. Because they are solvated, their movement drags the BGE in the same direction. EOF mobility μ_{EOF} (cm^2/Vs) is thereby estimated with **Eq. 1.11**:

$$\mu_{EOF} = \frac{\varepsilon\zeta}{\eta} \quad \text{(Eq. 1.11)}$$

With ε the dielectric constant of the BGE. ζ depends on the composition of the BGE, *i.e.*, its ionic strength. Indeed, increasing the ionic strength of the BGE leads to more counterions attracted to the capillary wall, a decrease of ζ, and, thus, a lower μ_{EOF}. The magnitude of EOF depends on the pH of the BGE. μ_{EOF} is the strongest at high pH where the silanol groups are predominantly depronated and close to zero at pH lower than 2-3 due to the protonation of the silanols. EOF presents a unique feature with a flat flow velocity profile which is uniform, on the contrary of the parabolic profiles observed in case of applied pressure, as in LC. Less zone broadening and better efficiencies can thus be obtained.

The apparent mobility of a solute μ_{app} is defined with **Eq. 1.12**:

$$\mu_{app} = \mu_{eff} + \mu_{EOF} \quad \text{(Eq. 1.12)}$$

The migration time t_m of an analyte is defined as the time required reaching the point of detection and is related to μ_{app} according to **Eq. 1.13**:

$$\mu_{app} = \frac{l_{eff}}{t_m E} = \frac{l_{eff} L_{tot}}{t_m U} \quad \text{(Eq. 1.13)}$$

Where l_{eff} and L_{tot} are the effective length (length until the point of detection) and the total length of the capillary, respectively. Rapid separations are therefore obtained with short capillaries and high voltages, provided that no excessive Joule heating is observed.

In CZE, the longitudinal (axial) diffusion is the predominant mechanism leading to peak broadening, while radial diffusion is negligible due to the flat flow velocity profile. The efficiency, expressed as the number of theoretical plates N, is estimated with **Eq. 1.14**:

$$N = \frac{\mu_{app} l_{eff} U}{2 D L_{tot}} = 5.54 \left(\frac{t_m}{w_{1/2}}\right)^2 = 16 \left(\frac{t_m}{w_{base}}\right)^2 \quad \text{(Eq. 1.14)}$$

With D the diffusion coefficient and $w_{1/2}$ and w_{base} the temporal peak width at half height and at baseline, respectively. Zone broadening in CZE depends on the longitudinal diffusion, the Joule heating, the injected volume, the adsorption of analytes to the capillary wall, the electrodispersion (*i.e.*, mismatched conductivities between sample and BGE), and the width of the detection window.

The resolution R of two analytes presenting a migration time of t_{m1} and t_{m2} and a baseline temporal peak with of w_1 and w_2, respectively, can be calculated with **Eq. 1.15**:

$$R = \frac{2(t_{m1} - t_{m2})}{w_1 + w_2} = \frac{\Delta\mu_{eff}\sqrt{U}}{4\sqrt{2D(\overline{\mu_{eff}} + \mu_{EOF})}} \quad \text{(Eq. 1.15)}$$

With $\Delta\mu_{eff}$ the difference of effective mobility between the two analytes, $\overline{\mu_{eff}}$ their average mobility, and D the average diffusion coefficient. According to **Eq. 1.15**, for two analytes with only a small difference in electrophoretic mobility, maximal resolution can be obtained when they migrate in opposite direction of the EOF, but at approximately the same absolute rate as the EOF [316].

In CZE, cations migrate towards the cathode, which is generally placed at the detector side, while anions migrate in the direction of the anode. In presence of significant EOF, anions and neutral species will move to the detector since μ_{EOF} is in most cases significantly higher than μ_{eff}. Neutral compounds will therefore be detected since they migrate with the EOF but cannot be separated due to their similar μ_{eff} close to zero. Neutral species can be separated using alternative CE modes, such as micellar electrokinetic chromatography (MEKC) or microemulsion electrokinetic chromatography (MEEKC). MEKC is achieved with the addition of surfactants to the BGE (*e.g.*, sodium dodecyl sulfate, SDS) which form micelles at a concentration above the critical micelle concentration. An additional separation for neutral and charged compounds occurs *via* hydrophobic interactions between analytes and the micelles which also have their own mobility and act as a pseudo-stationary phase. MEEKC involves the same principle, replacing the micelles by oil droplets usually composed of SDS, octane, and butan-1-ol. Octane is water-immiscible and forms small droplets, whereas SDS decreases the surface tension and butan-1-ol stabilizes the droplet. Partition occurs between the hydrophobic droplets and the poorly water-soluble analytes. Ion-pair interactions with the negatively charged droplet are also possible.

Other alternative CE separation modes can be envisaged if CZE does not provide a sufficient selectivity, *i.e.*, capillary electrochromatography (CEC), where the capillary contains a chromatographic stationary phase, capillary isoelectric focusing (CIEF), which involves pH gradients between the anode and the cathode, non-aqueous capillary electrophoresis (NACE), using non-aqueous BGEs for the separation, capillary gel electrophoresis (CGE), involving the size-based separation in a gel acting as a molecular sieve, and affinity capillary electrophoresis, which is used for the evaluation of molecular interactions [318-321].

The most commonly used and historical detection mode is UV/Vis optical detection which is directly performed through the capillary wall, provided the removal of the polyimide external coating. UV/Vis and DAD detectors are universal and, due to the on-capillary detection, they contribute to the high efficiencies observed in CZE. However, the selectivity UV/Vis detection is rather low, while the sensitivity is critical due to the short optical path length, corresponding to the i.d. of the capillary. Using extended path length flow cells which do not increase the overall capillary i.d. (which would lead to increased current and Joule heating) can be envisaged to improve the sensitivity, for example with bubble cells or Z-shape flow cells. An enhanced sensitivity is chiefly observed when hyphenating CE to other detectors such as fluorescence, laser-induced fluorescence, amperometry, and MS. Due to the high sensitivity and selectivity, the universality, and the potential structural information provided by MS, CE-MS remains the most powerful and promising combination for complex analytical challenges.

3.2 Fundamentals in CE-MS interfacing

The group of Smith was the first to present in 1987 a CE-MS coupling with the use of a metal sheath around the capillary tip to replace one of the electrodes of the conventional CE-UV setup, and ESI as the ionization source [322]. The first experiments were promising but pointed out the challenges in hyphenating CE with MS due to the use of non-volatile BGEs and the low CE effluent flows which were not easy to combine with ESI process.

3.2.1 Ionization sources

Numerous ionization methods have been evaluated for CE-MS combination. API sources such as ESI, APCI, and APPI remain the most widely used techniques due to their large range of applications, while inductively coupled plasma (ICP) and MALDI are only considered in specific applications. ICP involves the use of extremely high temperatures to completely fragment the compounds of interest and is mainly applied to the trace analysis of metals. MALDI requires the vaporization of the CE effluent with laser energy. CE fractions are generally first collected and deposited on a MALDI plate prior to the laser application. The MALDI light-absorbing matrix solution can also be used as BGE. Due to the extra efforts required to collect the CE fractions with frequent current losses and the costs of the equipment, CE-MALDI/MS remains sparsely used, often with home-made interfaces and mainly in the characterization of large biomolecules [323-325].

APCI is routinely used in LC-MS but is still limited for CE-MS coupling. Indeed, APCI is considered a mass flow sensitive ionization technique, limiting its use with the low flow rates observed with CE. The first attempts in CE-APCI-MS with lab-made hyphenation led to poor sensitivities [326]. A new set-up using an orthogonal LC-APCI source, a 36-mm long plastic spacer, and a commercial CE-MS sprayer originally designed for ESI process was proposed in 2009 and presented a significantly better sensitivity, leading to LODs in the range of µg/mL for a set of model compounds. Some improvements are still required by adapting the APCI source to the flow rates encountered in CE to further increase the overall performance [327].

APPI presents some advantages over APCI for CE-MS combination, particularly an improved sensitivity at low flow rates (< 50 µL/min) with lower background noise. Moreover, APPI is less prone to ion suppression than APCI and ESI, may be used for both polar and apolar compounds ionization, and appears to be not affected by the BGE composition allowing for the use of MEKC or MEEKC modes with MS [328]. A spacer is positioned between the sprayer and the nebulizer to adapt the LC-APCI sources to the CE sprayer, while the dopant can be added to the sheath-liquid in the sheath flow configuration [323,328-331]. However, APPI remains rarely used, and also requires some geometry adjustments to be fully adapted to low flow rates.

ESI remains the primary choice for on-line coupling of CZE with MS and can be applied to numerous fields, *e.g.*, low-molecular weight compounds, peptides, and proteins analysis. The use of volatile electrolytes such as acetate, formate, and ammonium is required in ESI due to the strong ion suppression encountered with phosphate or borate buffers. Moreover, selectivity modifiers such as micelles, microemulsions, or chiral selectors are not readily MS compatible due to their low volatility.

In these cases, different strategies can be implemented such as partial-filling techniques or the use of APPI source [323,324].

Numerous CE and ESI properties have to be considered for a successful CE-ESI-MS coupling. Indeed, two separate electrical circuits are involved, leading to potential issues in the coupling. First, the CE electrical circuit operates at currents in the µA range, while stable ESI process requires currents in the nA range. Moreover, high conductive BGEs used in CE separation are not recommended for ESI stability which requires solvents with low conductivity and low surface tension. Finally, CE effluents involve flow rates of hundreds of nL/min, which is not sufficient to allow for a stable spray with conventional ESI emitters [324]. These considerations and the different CE-ESI-MS interfaces are discussed in the next section.

3.2.2 CE-MS interfaces

3.2.2.1 Electrochemistry aspects

Two electrolytic cells are involved in CE-ESI-MS, *i.e.*, a first circuit composed CE inlet and outlet electrodes and a second formed with ESI and MS inlet electrodes. The CE outlet electrode simultaneously acts as the ESI electrode (ESI needle) and is thus shared by the two circuits. In positive ESI mode, oxidation of water takes place at the metallic interface of the ESI electrode (anode), and reduction occurs at the MS endplate (cathode), leading to gas bubble formation (hydrogen gas) and reduction of pH (hydroxide ions) up to 4.4 units in the ESI cone. Gas bubbles can lead to unstable CE-MS analyses due to fluctuations in CE and ESI currents. The reverse process occurs in ESI negative mode, where a higher number of analyte electrochemical reactions are observed, leading to higher background noise and decreased spray stability. Oxidative, reductive, or both processes can occur at the shared ESI electrode, depending on the polarities used in CE and ESI process. The electrochemical reactions related to the CE circuit are dominant on the one resulting from ESI circuit. Usually, CE with normal polarity combined with ESI in positive mode is the preferred combination, where the shared ESI electrode acts at the same time as a cathode for CE circuit and an anode for ESI circuit, and is prone to gas bubbles formation or pH decrease, especially with the use of high ESI voltages. Under reversed CE polarity and ESI positive mode, the shared electrode is anodic for both circuits. The needle material is prone to oxidation, which has been demonstrated with the release of Fe^{2+} and Ni^{2+} species from stainless steel needle, leading to the complexation with analytes (adducts formation), iron oxides clogging the capillary outlet, and corroded needle [332]. This issue can be overcome with the use of commercially available platinum needle instead of the conventional stainless steel one [236,324,333].

The electrical interfacing between CE and ESI-MS can be afforded with two different approaches. On the one hand, a high voltage is applied to the inlet of the separation capillary, and the outlet electrode is kept at ground. The electrospray can be then established with the application of a voltage at the MS inlet capillary, with entrance of negative ions in case of positive voltage (negative ion mode), and *vice versa*. This electrical interfacing is used by MS vendors like Agilent Technologies and Bruker Daltonics which provide instruments with the voltage applied at the MS entrance. This configuration allows for the use of high conductive BGEs (generating a CE current up to 50-60 µA,

depending on the subsequent Joule heating) without significant influence on the ESI process. On another hand, the shared CE/ESI electrode is set at high voltage, involving two different currents, *i.e.*, CE and ESI currents. These currents differ from at least two or three order of magnitude, meaning that the power supply for ESI generation has to sink a significant current from the CE separation and can be damaged by it. An adjustable resistor can be used between the shared electrode and ground to sink this current. Low-conductive BGEs are also recommended in this configuration for an undisturbed ESI process [333,334].

The conventional classification between CE-ESI-MS interfaces relies on the presence or the absence of a make-up liquid. Recently, Bonvin *et al.* proposed an alternative approach which distinguishes the interfaces according to the operating flow rate emerging from the CE outlet, *i.e.*, electrospray and nanospray regimes [236]; this classification will be used here.

3.2.2.2 Electrospray approaches

Interfaces based on the electrospray approach operate at flow rates comprised between 1 and 1000 µL/min and are often pneumatically assisted by a nebulizing gas to ensure a stable ESI current; they include the *sheath-flow* and the *liquid-junction* interfaces [236].

The coaxial sheath-flow interface is to date the most popular and commercially available coupling device. The commercial set-up consists of a triple-tube sprayer placed orthogonally in front of the MS entrance. The capillary is contained in a stainless steel or Pt needle tube which delivers the so-called *sheath liquid*, surrounded by another stainless steel concentric tube providing the nebulizing gas. The latter is used to help in the desolvation and provides the cooling of the CE capillary [324]. The sheath-flow interface is illustrated in **Fig. 1.10**.

The sheath liquid is composed of a hydro-organic mixture providing appropriate solvent conditions for ionization and evaporation. A 50:50 (*v/v*) proportion is generally preferred to allow for satisfactory spray stability with sufficient conductivity. Mixtures composed of methanol or isopropanol with water are usually selected, isopropanol showing a slightly higher ionization efficiency as well as solvent purity. Acetonitrile can lead to a swelling of the polyimide layer in case of long-term contact, deteriorating the separation or even clogging the capillary [324]. A small percentage of volatile acid or base is generally added to the mixture to enhance the ionization of the compounds. Formic acid may be preferred to acetic acid due to less ion pairing, while ammonia is prone to adducts formation [335]. The concentration of this electrolyte should not be too high due to ESI currents instability. The sheath liquid is delivered at a flow rate between 1 and 10 µL/min, providing a sufficient flow rate for stable ESI process, a decrease in surface tension of the sprayed liquid, and the dilution of high-concentrated BGEs. Besides the sheath-liquid composition, operating parameters such as the nebulizing gas, drying gas, and sheath-liquid flow rates have to be carefully optimized to obtain the higher intensities. The position of the capillary tip within the needle is of utmost importance to ensure stable analyses and should present a protrusion of *ca.* 0.1-0.2 mm with a regular cut [324,336]. Once the operating conditions and capillary position optimized, the coaxial sheath-liquid interface presents a satisfactory stability and robustness, as well as ease of operation, which allows to perform multiple and repeatable analyses in sequence mode. Moreover, decoupling the CE and ESI processes offers to select the best conditions for the separation and the ionization.

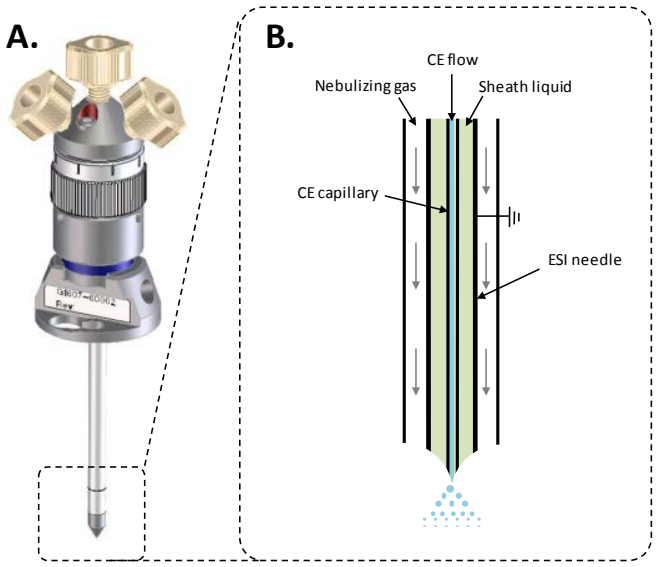

Figure 1.10. Conventional sheath-flow interface. A. Commercial CE-ESI-MS sprayer (courtesy of Agilent Technologies), B. Illustration of the triple-tube configuration. Adapted from [236] and [325].

Three major drawbacks have been reported with the coaxial sheath-flow interface [323,324,333]:

(i) The introduction of a dilution factor due to the relatively high flow rate of the sheath liquid compared to the CE effluent. However, the sheath liquid being volatile and evaporated during the ESI process, the dilution might not affect in proportion the sensitivity.

(ii) Increasing the flow rate of the nebulizing gas causes a parabolic flow within the CE capillary due to the so-called *suction effect* occurring at the capillary terminus, which leads to decreased efficiency and resolution. A compromised value has to be determined taking into account the needs in resolution, speed of analysis, and ionization intensities. A higher protrusion of the CE capillary out of the needle can help lower the suction effect.

(iii) Using different sheath liquid and BGE compositions can lead to a disturbed buffer system at the sprayer's tip due to the *moving ion boundary effect*. In normal CE polarity, anions of the BGE are expelled by the CE capillary and replaced by anions coming from the sheath liquid to ensure the electroneutrality [337]. This moving ion boundary effect is particularly significant with low EOF and affects the pH and the conductivity of the BGE, leading to a constant decrease of CE current and unstable CE-MS analyses. Moreover, the separation resolution can be impaired due to this mechanism [324]. Using coated capillaries (anionic or cationic coating) providing a high EOF can be used to circumvent this issue when using acidic BGEs at very low pH values (≤ 2). Furthermore, selecting the same electrolyte for BGE and sheath liquid composition is recommended.

Liquid-junction interfaces were first developed to overcome the dilution encountered with the sheath-flow interface. In this configuration, the sheath liquid is delivered *via* a tee junction through a 20- to 200- µm gap between the end of the capillary and the needle, providing the electrical

connection and decoupling CE from ESI process. This interface has shown some interest but can lead to peak broadening due to the dead volumes encountered and if the alignment between the capillary outlet and the needle is not carefully optimized. It is thus not as robust as the coaxial sheath-flow interface and is yet not commercialized.

3.2.2.3 Nanospray approaches

Nanospray regime is typically observed at flow rates between 1 and 1000 nL/min, depending on the i.d. of the emitter. The formed droplets are smaller than *sub*-µm diameter, leading to a facilitated evaporation of poorly volatile solutions and a fast and efficient generation of gas-phase ions. Low emitters i.d. is required to deliver low flow rates and ensure the stability of the Taylor cone. The emitter is positioned close to the MS entrance, allowing for an enhanced ion sampling by the MS compared to the electrospray approaches. The nanospray regime is considered a mass flux-sensitive response at flow rates of *ca.* ≤ 100 nL/min, depending on the i.d. of the emitter. *Sheathless* and *low-flow* interfaces are based on this nanospray approach [236].

In the sheathless approaches, no dilution effects occur, leading to higher sensitivities than sheath-flow interfaces. Numerous sheathless interfaces have been proposed since the first development in 1987 by Oliveira *et al*. The electrical contact between CE and MS is generally achieved with a transformation of the CE capillary outlet into a conductive tip. This can be done by numerous methods, such as the application of conductive coating to the emitter tip (*e.g.*, gold, silver, copper, *etc.*), the insertion of a wire at the tip or through a hole, or the use of porous and etched capillaries in a metal sleeve [333]. However, the manufacture process of this tip is very tedious and poorly reproducible; the proposed sheathless interfaces thereby mainly remained at the experimental stage. The only prototype that will likely be commercialized is based on a sheathless interface proposed by Moini in 2007 [338]. In this interface, a 49% hydrofluoric acid solution is used to etch the capillary outlet on a 2.5- to 3.8-cm section, producing a thin porous tip. The capillary i.d. remains the same (30 µm), while the external diameter is significantly reduced. The electrical connection is achieved by inserting the capillary tip into a grounded ESI needle (or metal sheet) until a *ca.* 2-mm protrusion. The needle is filled with a conductive liquid, whose ions diffuse through the wall pores, providing the electrical contact. This interface, illustrated in **Fig. 1.11.A**, presents some advantages, such as an automated manufacture, the possibility of cutting a small section of the tip if clogged, and the minimization of detrimental electrolyte reactions at the tip. This prototype has been further developed by Beckman Coulter under the trademark name *CESI-MS technology* with the use of an OptiMS cartridge assembly consisting in the separation capillary, the conductive liquid transfer line, and the sprayer housing. The OptiMS cartridge is shown in **Fig. 1.11.B**. When plugged into specific nanospray source adapters, this OptiMS cartridge can be used for hyphenating CE to AB Sciex, Bruker, ThermoFisher, and Waters MS instruments. The prototype has shown to display a mass flux-sensitive response when working with flow rates below 30 nL/min and concentration-sensitive response with higher flow rates [339]. Careful selection of BGE conditions and optimization of the position of the sprayer tip in front of the MS (xyz adjustments) are required to ensure stable analyses. Although having already been evaluated and shown interesting performance in different laboratories, *i.e.*, higher sensitivity and resolution than the widely used sheath-flow interface, this CESI-MS technology is currently still not commercially available and seems to be significantly less

robust, which currently hinders its use in routine analysis [340]. With the very recent announcement (July 2013) of the transition of the CE technologies of Beckman Coulter Life Sciences to AB Sciex (both owned by Danaher Corporation), a rapid commercialization of this new CE-MS technology is expected.

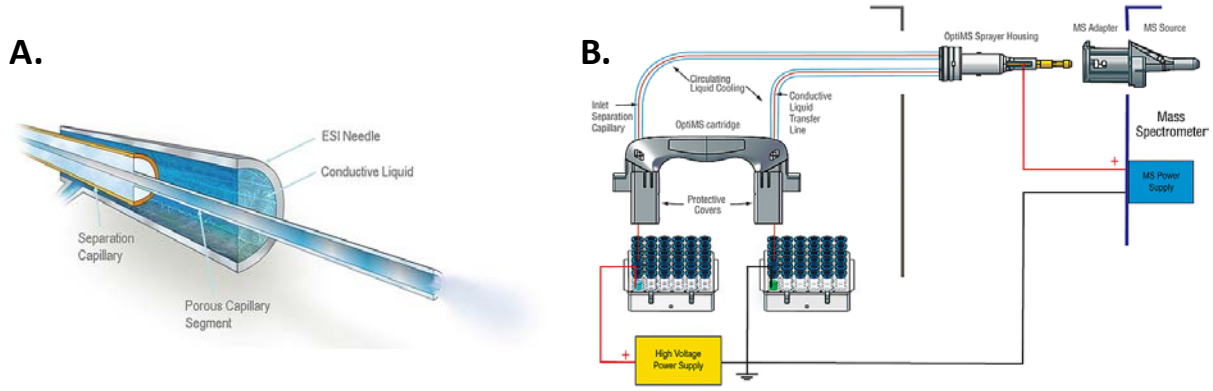

Figure 1.11. OptiMS technology provided by Beckman Coulter. A. Schematic of CESI sprayer, B. OptiMS cartridge assembly. Courtesy of Beckman Coulter, Inc.

Other developments were also performed in low sheath-flow interfaces with the use of a make-up liquid flowing at very low rates, for example with pressurized liquid junction or junction-at-the-tip interfaces. The most promising work was proposed by Zhong, Maxwell, and Chen, with an improved junction-at-the-tip interface with beveled tip design [341-343]. In this design, the capillary is inserted into a stainless steel emitter needle presenting a beveled tip. The space enclosed between the capillary outlet and the inner needle surface forms an open flow-through microvial, as shown in **Fig. 1.12.A**, which acts as an outlet vial and a terminal electrode. An additional grounded, pressurized reservoir supplies a chemical modifier solution at low flow rate to the tip of the capillary to ensure a stable electrospray with minimal dilution, as shown in **Fig. 1.12.B**. This interface is not yet commercialized but has shown interesting performance in terms of sensitivity and robustness, whereas maintaining the CE laminar flow within the microvial and, thus, avoiding peak broadening [341-343]. Another promising low-flow approach has been proposed by Dovichi and co-workers with the separation capillary placed inside a tapered glass emitter. In this interface, the low-flow sheath liquid is driven by electroosmosis and flows over the end of the capillary, mixing with the CE effluent and closing the circuit [344].

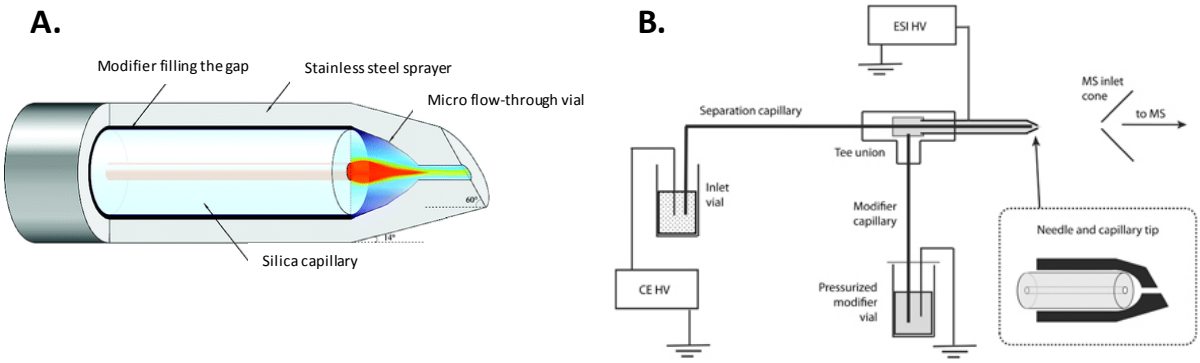

Figure 1.12. Junction-at-the-tip interface proposed by Zhong, Maxwell, and Chen. A. Schematic of the open flow-through microvial, B. Illustration of the interface apparatus. Reprinted from [343] and [341].

3.2.3 Mass analyzers

All mass analyzers can be hyphenated to CE and numerous CE-MS combinations have been already used in miscellaneous bioapplications. Most research was performed using quadrupole mass analyzers, QqQs, and ITs for qualitative or quantitative analysis. The emergence of HRMS led to further improvements with successful combination of CE with TOF, QTOF, and even FT-ICR and Orbitrap© instruments [323,324,345,346].

The selection of mass analyzer as well as the acquisition mode also relies on the efficiencies obtained during the CE separation. Indeed, due to the narrow peaks observed in CE (few s), a fast MS acquisition is required. In case of quadrupole and QqQ detection, SIM and SRM are preferred to the full-scan mode due to the slow duty cycle of the latter (> 1 s). Previous generations of quadrupole instruments presented dwell times (*i.e.*, time required to collect the data for each *m/z*) and inter-channel delay (*i.e.*, time required to switch from one to another *m/z*) of *ca.* 20 to 100 ms. Recent commercial quadrupoles allow for a decrease of both dwell times and inter-channel delays down to 5 to 10 ms [253]. The same improvements have been observed for QqQ instruments, which nowadays present dwell times and inter-channel delay as low as 1 to 5 ms. Moreover, recent instruments allow for a time-segmentation of the electropherogram, with the acquisition of different SRM data within defined time windows, which is particularly advantageous in multi-analytes determination.

The high mass accuracy, sensitivity, and resolution provided by HRMS led to a significant increase in their coupling with CE over the last years in bioanalysis. Recent TOF and QTOF mass analyzers present high acquisition speeds with 10-40 spectra/s. CE-TOF/MS with sheath-flow or sheathless interfaces has seen an enhanced number of publications over the last five years which were mostly concerned with intact protein analysis or metabolomics [347]. CE-QTOF combination is also emerging and will probably be more regarded the next few years for proteomics or metabolomics approaches [325]. FT-ICR and Orbitrap© both remain sparsely used and only in protein characterization due to their limited data acquisition, *i.e.*, 1 scan/s for the FT-ICR and 1 spectrum/s at highest resolution for the Orbitrap©, as well as their impressive costs.

Therefore, the selection of the mass analyzer for CE hyphenation mainly relies on the field of application (low-molecular weight compounds *versus* proteins, qualitative *versus* quantitative determination) but also on the sensitivity, resolution, data acquisition rate, mass accuracy, and instrument costs of the analyzer [335].

3.3 Retrospective and current role of CE-MS in clinical and forensic toxicology

Among the already mentioned advantages of CE-MS, some of them are particularly interesting for forensic and clinical applications, *i.e.*, (i) the low sample consumption, for example favorable in post-mortem analysis or newborn screening, (ii) the low solvents and reagents consumption, having less negative impact on the environment and the operator's health, (iii) the low costs associated with previous item and the low prices of capillaries, and (iv) the speed of analysis, including pre-conditioning and rinsing steps.

Since the first attempts of CE-MS in bioanalysis almost twenty years ago, CE-MS applications in forensic and clinical toxicology has been continuously slightly increasing, starting from an average number of publications of 10-20 *per* year to *ca.* 50 papers annually published in this decade [346]. The published papers concern *inter alia* proteins analysis in body fluids (*e.g.*, doping control or other forensic cases) as well as targeted analyses of several classes of different drugs or pharmaceuticals and their respective phase I and/or phase II metabolites. CE-MS has also been frequently used for chiral determinations due to the powerful separation obtained for enantiomers [348-358].

Very few CE-MS methods have been developed for general unknown screenings. The most relevant are the development of a CE-TOF/MS screening method for hair, blood, and urine analysis followed by compounds identification with the help of free access database by Polettini *et al.* [359] and a CE-TOF/MS procedure for a broad spectrum screening in hair samples by Gottardo *et al.* [360]. Compounds quantitation with CE-MS is more widespread, including for example the quantitation of oxycodone and its metabolites in urine [361], methylphenidate in urine [362], drugs of abuse and metabolites in hair samples [363], GHB in urine [364], enantiomeric drugs in plasma [365], 2C-T series designers drugs in plasma [366], and ecstasy and methadone in plasma [367]. However, only few of the existing methods were fully and systematically validated according to the reference guidelines. The validation procedure is often incomplete, mainly regarding the correct estimation of LLOQ and the evaluation of matrix effects. Only Schappler *et al.* studied the matrix effects with post-column infusion experiments for the quantification of ecstasy and methadone in plasma [367]. CE-MS is as much prone to ion suppression or enhancement as LC-MS, thereby rendering notably advised the evaluation of matrix effects to ensure an accurate quantitation. CE-MS method validation should be done according to the reference procedures used for LC-MS purposes, even if some validation criteria would clearly deserve individual specificities, one of the best examples being the repeatability in migration times. At present, none of the official guidelines includes or recommends the use of CE-MS in STA or for quantitative purposes.

Interestingly, a greater attention has been paid over the last few years on the use of CE-MS in metabolomics and the discovery of clinically relevant biomarkers, with the development of numerous CE-TOF/MS procedures for the profiling of urine, cerebrospinal fluid, or other body fluids. It is worth mentioning that the analytical strategy implemented for untargeted metabolomics studies by CE-MS is similar as procedures developed for general unknown screenings. Hopefully the progresses made in CE-MS for metabolome analysis will be beneficial for the development of CE-MS tools for clinical and forensic toxicology in the near future, including sample pre-treatment, analysis, and data treatment.

In 2006, in their first retrospective study of the recent advances in the application of CE to forensic sciences, Tagliaro and Bortolotti concluded their manuscript with the following statement: “(…) the major improvement of CE is represented by the now robust and commercially available coupling with MS, which fulfills the most strict requirement of forensic toxicology in terms of analytical accuracy” [357]. Few years later, in 2012, in their fourth retrospective study, this ambitious and optimist statement was replaced by less enthusiastic comments: “(…) the pace at which CE is finding application in forensics is much slower than one could envision. The reasons this phenomenon, in our opinion, mostly lay in the background knowledge required for a proper use and application of CE (…). Unfortunately, the majority of forensic chemists have no or only superficial experience in

electrophoretic techniques, (…) [and] almost ignore the fundamentals of instrumental analysis. So, CE desperately needs a new type of "holistic analyst" (a hybrid of analytical chemist and biochemist) (…) to face the need of CE and related technologies" [355].

3.4 Future challenges

Among the persistent raised drawbacks encountered with CE-MS, the relatively low sensitivity, repeatability, and ease-of-use remain the most predominant. Despite the combination of CE with MS, presenting a significantly enhanced sensitivity compared to CE-UV configuration, the injected sample volumes remains extremely low (*ca.* 10-20 nL) compared to LC-MS (*ca.* 2-10 µL). This difference clearly weights against the use of CE-MS in bioanalysis.

The development of nanospray-based ionization approaches was really promising to provide up to 100-fold improvement in sensitivity compared to the sheath-flow configuration. However, at present, no robust and stable system is commercially available. Prototypes and lab-made devices can be hardly implemented in clinical or forensic laboratories for routine analysis. The most stable configuration for hyphenating CE with ESI-MS remains the sheath-flow interface. This system has shown to be stable and robust in numerous studies, with the possibility to perform overnight sequences. Once the basic CE knowledge assimilated, the hyphenation explained and managed, and the source conditions optimized (mainly the position of the capillary tip protruding out of the sprayer needle), the CE-MS system is easily accessible for LC-MS or GC-MS-trained analysts and technicians for routine analysis. As for LC-MS and GC-MS, CE-MS requires regular cleaning and instrument maintenance (*e.g.,* electrodes and needle cleaning) which can be easily implemented.

Innovative developments in the geometry of the sheath-flow interface have already started with the commercialization of a new sprayer shape which is more adapted to CE-MS configuration, as discussed in **Chapter III**. The next years will likely see the emergence of ESI sprayers and sources geometries dedicated to the low flow encountered with CE-MS. This will hopefully provide significant improvements in the sensitivity while keeping the robustness of this configuration.

At present, CE-MS can be nonetheless implemented in toxicology by using an alternative approach which consists in considering the whole analytical process, and not only the separation or detection part. As illustrated in **Fig. 1.13**, an analytical procedure is composed of multiple sequential stages, ranging from the sample preparation, the analytes separation, their ionization, the detection, and finally the data treatment. Each step can be prone to improvements to achieve a maximized performance for the whole process.

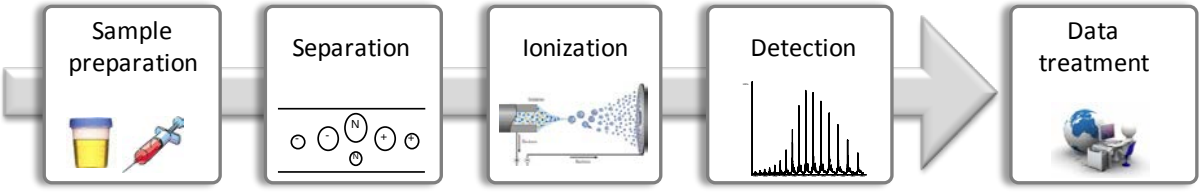

Figure 1.13. CE-MS analytical process. The analytical process encompasses multiple steps, from the sample preparation to the data treatment.

This strategy was adopted for the studies presented in this thesis, aiming at proposing relevant improvements at each step of the process. The lower sensitivity encountered in CE-MS compared to GC-MS and LC-MS being the most relevant issue, this thesis focuses on strategies to improve the overall sensitivity, *e.g.*, by using adapted sample preparation procedures, on-line sample preconcentration, or highly sensitive mass analyzers. By enhancing the whole performance of the CE-MS process, the analytical sensitivity can be significantly improved, as well as the diagnostic sensitivity and specificity in screening approaches, leading to a lower number of false-negative and false-positive results, respectively.

4 Conclusions

Each analytical technique presents advantages and drawbacks. Even with the most recent technology improvements, for example with the emergence of UHPLC instruments or HRMS, LC-MS and GC-MS are no exception. One single analytical technique is not enough to provide full answers to the complex analytical issues and challenges encountered in bioanalysis. Moreover, two-step workflows are most of the time required by reference guidelines, highlighting the need of orthogonal and alternatives techniques.

The purpose of the present manuscript is not to promote CE-MS as a “gold standard” technique instead of the LC- and GC-based techniques, but aims at proposing an alternative analytical tool that can be used in addition to the formers. CE-MS likewise presents some advantages and drawbacks. The reported studies show the potential of CE-MS in toxicological applications when the technique is fully invested by enhancing the overall performance. CE-MS is hereafter proposed as a competitive alternative and orthogonal technique that may be envisaged for general unknown or multi-target screenings; or for quantitative or confirmatory analysis. The proposed procedures may be also advantageous when few quantities of sample are available or to lower the generated costs.

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Chapter II.

Chapter II. Emergence of novel sample preparations

1 Introduction

Green chemistry is a concept that emerged in the late 1990s and is defined as the use of chemistry techniques and methodologies reducing or eliminating the utilization or generation of feedstock, products, by-products, solvents, and reagents that are hazardous to human health or the environment [1]. Based on this concept, the European Community Regulation on chemicals and their safe use (EC 1907/2006) dealing with the registration, evaluation, authorization and restriction of chemical substances (REACH) was created in 2006 and aims *inter alia* at providing a high level of protection of both human health and environment. This consideration was also raised in the *Twelve Principles of Green Chemistry* edited by Anastas and Warner, summarized as following [2,3]:

(i) Prevent waste
(ii) Maximize the incorporation of all materials into the final product
(iii) Use and generate less hazardous chemical syntheses
(iv) Design safer chemical products
(v) Use safer solvents and other auxiliary substances
(vi) Minimize the energy requirements, preferring synthetic methods at ambient temperature and pressure
(vii) Use renewable raw material or feedstock
(viii) Minimize unnecessary derivatization to avoid additional reagents generating waste
(ix) Use catalytic reagents instead of stoichiometric reagents
(x) Design the chemical products which present an innocuous degradation and do not persist in the environment at the end of their function
(xi) Prevent the pollution with real-time analytical methodologies
(xii) Minimize the potential of chemical accidents, including releases, fires, and explosions.

These twelve principles were mainly related to the chemical and pharmaceutical industry, focusing on green organic synthesis processes. Only some of them, *i.e.*, the first, fifth, sixth, and eighth can be fully applied in analytical chemistry. The concept of *green analytical chemistry* (GAC) emerged in 2000, concerned with the role of analytical chemists in making laboratory practices more environmentally friendly [4,5]. The key goals that should be achieved according to the GAC concept is to reduce or eliminate the use of solvents and other chemical substances in the sample pre-treatment and the measurement step, minimize the consumption of energy, have a proper management of waste, and increase the safety of the operator [4,6]. The latter is particularly important due to the potential acute or chronic toxicity or organic solvents *via* inhalation or dermal exposure. Since inhaled solvents are introduced into the circulation *via* the lung alveoli, they may be distributed in the whole body prior to metabolism by the liver and excretion. Some deleterious effects on the organism are the depression of the central nervous system activity (from reduced function capacity to coma and death), *e.g.*, with dichloromethane or chloroform; a distal axonal peripheral neuropathy, *e.g.*, with hexane; the membrane and tissue irritation in case of skin contact, *e.g.*, with alcohols; a potential carcinogenicity, *e.g.*, with benzene and carbon tetrachloride; and

blindness or ototoxicity *via* ingestion, *e.g.*, with methanol. Acetonitrile is suspected by several agencies to be carcinogenic [7].

Based on a decade of reflection, the group of Namiesnik proposed this year the definition of new principles focused on GAC. They kept the four principles of Anastas and Warner that were already applicable in analytical chemistry and added eight points, leading to the following *Twelve Principles of GAC* [6]:

(i) Direct analytical techniques should be applied to avoid sample pretreatment
(ii) Minimal sample size and minimal number of samples are goals
(iii) *In situ* measurements should be performed
(iv) Integration of analytical processes and operations saves energy and reduces the use of reagents
(v) Automated and miniaturized methods should be selected
(vi) Derivatization should be avoided
(vii) Generation of a large volume of analytical waste should be avoided and proper management of analytical waste should be provided
(viii) Multi-analyte or multi-parameter methods are preferred *versus* methods using one analyte at a time
(ix) The use of energy should be minimized
(x) Reagents obtained from renewable source should be preferred
(xi) Toxic reagents should be laminated or replaced
(xii) The safety of the operator should be increased.

Important components of the analytical process having a significant impact in the aspect of GAC are illustrated in **Fig. 2.1**. A so-called *Eco-Scale* has been also proposed by the same group to evaluate the greenness of an analytical procedure, encompassing the six components of **Fig. 2.1**. This Eco-Scale classifies the different steps of the analytical procedure in the *greenest* option, a *medium-green* option, and a *not-green* option, taking into account the amount of solvents, their toxicity, the required energy, the occupational hazard, and the produced waste [8].

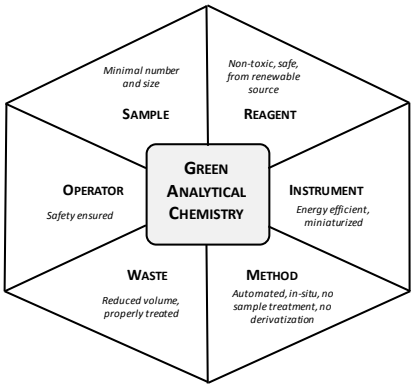

Figure 2.1. Schematic representation of the important components in the aspect of GAC. Adapted from [6].

According to the **Fig. 2.1**, CE is one of the greenest analytical tools due to the few amounts of the sample required, the use of aqueous BGEs, the miniaturization of the separation due to the capillary format, the possibility of direct injection of some biofluids, the reduced or no volume of waste

generated (mainly aqueous), and the safety for the operator. When using aqueous BGEs, the only source of health toxicity in CE is the preparation of the buffers and the manipulation of strong acids or bases. Daily practice does almost not require any organic solvents. Only methanol is necessary to condition the capillary, but few µL are used. Coupling CE to MS leads to a slight increase in organic solvents consumption due to the presence of the sheath liquid. However, with a flow rate of 1-10 µL/min, few mL are consumed *per* day, and only solvents with relatively low toxicity are used.

As exposed in **Chapter I**, in many clinical and forensic cases, biosamples cannot be directly injected or only diluted, and a sample preparation step is frequently required. This is also true for CE-MS, as highlighted in a study presented in **Appendix I**, where the direct injection of diluted urine provided LODs higher than 1 µg/mL for a large range of toxicological compounds. In order to assess lower concentrations, widely encountered in toxicology, compounds were extracted by LLE or SPE, leading to more acceptable LODs as low as 10 ng/mL.

Besides the lack of automation of many conventional sample preparation techniques and the multiple sources of analytical errors encountered with off-line procedures, the main drawback of this step is the high consumption of organic solvents which generally present a relatively high toxicity for the operators and the environment. Therefore, with the advent of the green chemistry concept, different sustainable approaches were proposed to reduce, replace, or avoid the use of organic solvents in sample preparation. Three interdependent strategies are envisaged when developing environmentally sustainable sample pre-treatments [5,9-11]:

(i) The implementation of solventless procedures (*e.g.*, head-space analysis, extraction with gas or thermal desorption, *etc.*);
(ii) The substitution of organic solvents by less-toxic alternatives, such as supercritical fluid extraction, cloud point extraction, subcritical water extraction, or ionic liquids (ILs); and
(iii) The use of microextraction techniques (MEs).

These three items can be combined to obtain even more sustainable techniques, *e.g.*, using MEs with non-toxic solvents. Depending on the analytical technique used downstream, a green sample preparation procedure can be classified in the solventless procedures or in MEs. An example is SPME, which does not require any solvent when coupled to GC-MS, but is classified in MEs in this manuscript when used with CE-MS due to the few quantities of solvent required for the liquid desorption.

2 Microextraction techniques

2.1 Principles

MEs, also referred to as *micro-sample preparation techniques*, encompass extraction procedures which significantly reduce the volume of extracting solvents while simultaneously decreasing the sample volume, the extraction time, and the operating costs. The definition of ME also involves the use of a relatively larger volume of sample compared to the extracting phase, which is normally not the case with conventional extraction techniques [12]. MEs can be classified according to their

extraction principle into two categories, *i.e.*, liquid-based MEs and solid-based MEs, according to their principle based on LLE or SPE, respectively.

2.2 Liquid-based microextractions

Liquid-based MEs are also termed *solvent microextractions*. The first liquid-based ME, referred to as *liquid-phase microextraction* (LPME), was proposed in 1996 by two groups which used a solvent drop in the µL range as extractant, *i.e.,* a 8-µL drop of *n*-octane by Cantwell an co-workers, and a 1.3-µL drop of chloroform by Dasgupta and co-workers. All the current developed methods are based on the LPME principle. A large variety of liquid-based MEs have been then developed, leading to more than one hundred terminologies currently found in the literature related to a technique derived from LPME [13]. This makes particularly difficult the classification of liquid-based MEs and their connection. Furthermore, numerous developed techniques are incorrectly referred to as LPME. Many of the liquid-based MEs have been used in combination with CE analysis and have been reviewed in the **Article I**. In this article, a classification based on their extraction principle and the improvements in extraction performance has been proposed and is summarized in **Fig. 2.2**. Liquid-based MEs all derive from either single-drop microextraction (SDME), in which a drop of organic solvent is suspended from a tip of a device and immersed in the sample, or hollow-fiber liquid-phase microextraction (HF-LPME), composing a 2-phase or 3-phase extracting system where a hollow polymeric fiber is used as a support for the organic or aqueous acceptor phase [14].

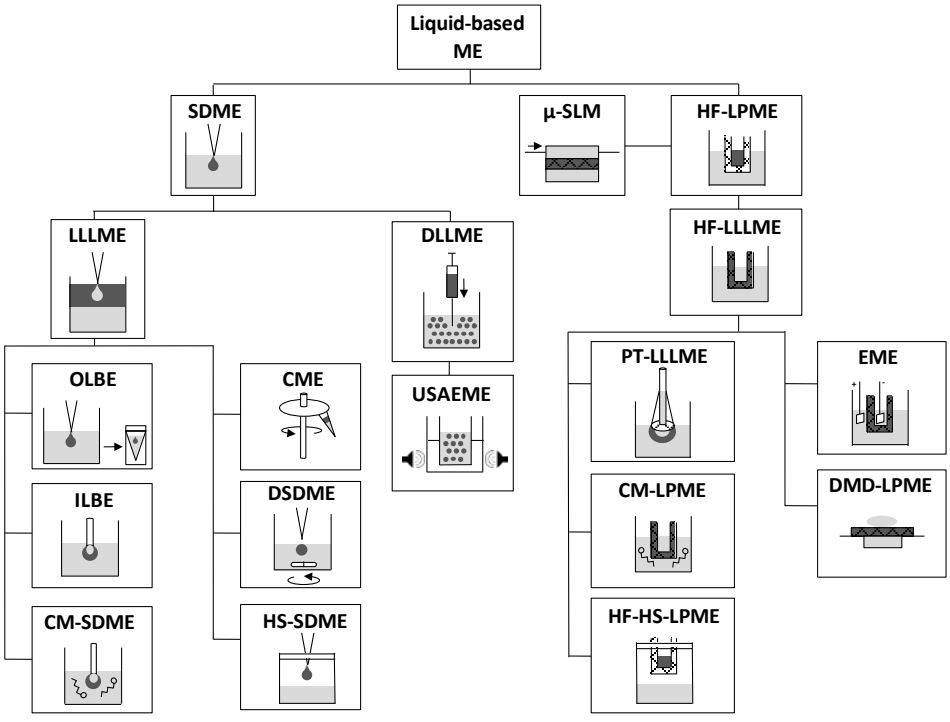

Figure 2.2. Liquid-phase microextractions. µ-SLM, micro-supported liquid membrane; CME, centrifuge microextraction; CM, carrier-mediated; DLLME, dispersive liquid-liquid microextraction; DMD, droplet-membrane-droplet microextraction; DSDME, directly suspended droplet microextraction; EME, electro membrane extraction; HF, hollow-fiber; HS, headspace; ILBE, in-line back-extraction; LLLME, liquid-liquid-liquid microextraction; LPME, liquid-phase microextraction; OLBE, on-line back-extraction; SDME, single-drop microextraction; USAEME, ultrasound-assisted emulsification microextraction. Reprinted from [14].

In a review covering a two-year period between mid-2010 and mid-2012, more than 200 papers devoted to liquid-based MEs were identified [13]. The vast majority of them were developed in

academic laboratories and are hardly applied in routine laboratories. Almost two-third of the studies published within these two years concerned extraction techniques based on the dispersive liquid-liquid microextraction (DLLME). DLLME is very simple and provides some additional advantages compared to the other liquid-based MEs such as an almost immediate extraction and high extraction recoveries. **Article II** concerns the development of a DLLME procedure combined with CE for the extraction of drugs of abuse in urine. DLLME is one of the only MEs that saw innovative and significant developments since its first occurrence to improve the procedure, most importantly in the feasibility in automation, as discussed in **Section 3**.

2.3 Solid-based microextractions

The first solid-based ME was proposed in the early 1990s with SPME. In SPME, a small amount of sorptive and non-porous extracting phase is coated on (*fiber SPME*) or inside (*in-tube SPME*) a solid support and exposed to the sample for a specific period of time. If the time is long enough, equilibrium is reached between the matrix and the extracting phase. Therefore, SPME and related techniques are not exhaustive techniques and only a proportion of the total quantity of analytes is extracted [15,16]. Many sorbents are commercially available and can be selected according to the polarity of the compounds to be extracted; sorbents such as RAM, molecularly-imprinted polymers (MIP), or monoliths are also available in micro-formats. All the developments in solid-based MEs rely on the coating of the sorbent on fibers, suspended particles, stirrer, disk, vessel walls, tubes, or the dispersion of the extracting phase within the sample. Besides SPME and derivatives, most relevant solid-phase MEs are the micro-extraction by packed sorbent (MEPS), where the extracting phase is coated within a syringe, and the stir-bar sorptive extraction (SBSE), which involves the use of stir-bars coated with polydimethylsiloxane or other sorptive extraction phases. As SPME, SBSE is a non-exhaustive extraction technique. Solid-based MEs combined with CE were reviewed in **Article I** and are illustrated in **Fig. 2.3**.

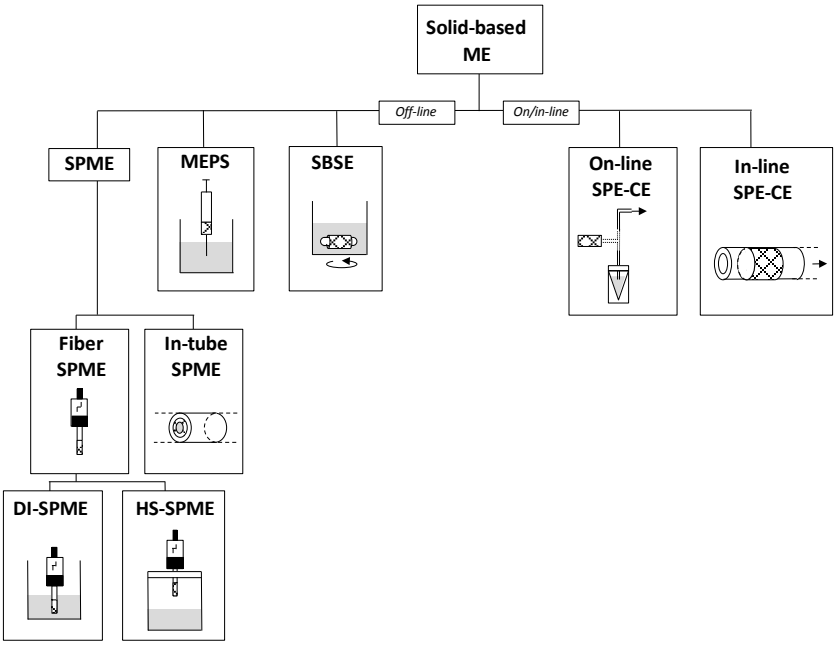

Figure 2.2. Solid-based microextractions. DI, direct immersion; MEPS, microextraction by packed sorbent; SBSE, stir-bar sorptive extraction. Reprinted from [14].

SPME, MEPS, and SBSE can be successfully used in combination with CE and provided some interesting applications in bioanalysis [14]. More than half of the studies reviewed in **Article I** involved the use of in-line or on-line SPE-CE procedures, as discussed in the next Section.

2.4 Coupling microextractions with capillary electrophoresis

2.4.1 Benefits of the combination

Besides the sustainable aspect provided by the very low amounts of organic solvents used, the combination of MEs and CE presents some advantageous characteristics. Organic extracts are generally evaporated before their reconstitution in a suitable solvent (acidified or basified water) and injection. Using EK injection prior to CE analysis allows for a direct injection of the organic extract, providing an important gain of time and limited loss of analytes that can be observed during the evaporation step. More importantly, the reconstitution of the dried extracts in a very small volume (20-30 µL) leads to substantial preconcentration factors which enhance the overall sensitivity. It has to be noticed that in case of 3-phase MEs, the acceptor solution is often at extreme pH or/and high ionic strength and using a low conductivity BGE will lead to peak broadening due to anti-stacking effect. Therefore, BGEs with high ionic strength are recommended for the analysis of 3-phase MEs extracts [17].

Fully automated systems with in-line or on-line configuration have raised the interest for both liquid-based and solid-based MEs. An at-line configuration, where a robotic arm is added or the replenishment system modified, involves the same solvent quantities than conventional sample preparations and thus presents a limited interest. In on-line coupling, an interface (vial, valve, or T-piece type) is used to connect the extracted stream or acceptor solution to the capillary; both extraction and CE process are performed independently. Finally, in-line procedures involve the direct contact of the acceptor solution with the capillary (liquid-based MEs) or the coating of a portion of the capillary with the SPE material. In-line couplings provide a complete analysis of the whole extract [17,18]. At-line, in-line, and on-line combinations of liquid-based MEs with CE are illustrated in **Fig. 2.4**, with the example of HF-LPME. Currently, only HF-LPME and membrane-based liquid-phase MEs can be directly coupled to CE.

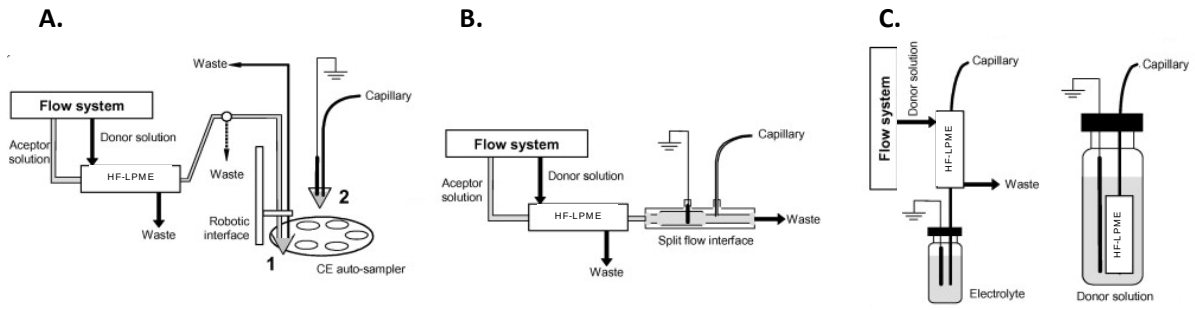

Figure 2.4. Direct combinations of HF-LPME to CE. A. At-line, B. On-line, and C. In-line. Adapted from [17].

On-line and, mainly, in-line SPE-CE represent the most promising ways of automated combination of solid-based MEs with CE [18-21]. The basic principle of these combinations is shown in **Fig. 2.5**. No

evaporation step is required with a direct injection of the extracts, providing an important gain of time. In on-line SPE-CE, the SPE column is not involved in the CE separation and does not disturb the electrophoretic process, however, only a portion of the eluate is injected. Chemically inert and electronically insulating materials are used for the interface to prevent electrochemical reactions and avoid current leakage *via* bubble formation. In in-line configuration, the SPE column is completely integrated to the capillary; conditioning solution, sample, washing, and eluate pass through the capillary. Only few nL are used to desorb the analytes and preconcentration factors higher than 100 can be reached. This desorption volume can still be too high to be injected without peak broadening and an on-line sample stacking is thus often performed to achieve good efficiencies. As the separation voltage is applied through the sorbent, some electrophoretic disturbances can be observed, depending on its length. Very complex samples can also lead to the same issue. SPE material can be coated in an open-tubular preconcentration capillary connected to the CE capillary using sleeves, a packed bed in the capillary retained by frits, or a thin impregnated membrane positioned between two capillaries (**Fig. 2.5.B**) [18,21]. C_{18}, C_8, monoliths, styrenedivinylbenzene, MIP-based sorbents, and antibodies (immunoaffinity extraction) have been already successfully used for in-line extraction. Silica- and polymer-based monoliths, despite not widely used yet, are particularly interesting as they can be prepared by *in situ* polymerization within the capillary, thus not requiring any frits [18]. In-line SPE-CE shows promising advantages such as its implementation in conventional CE system, the high preconcentration factors, the absence of dead-volumes, and the analysis of the complete eluate. Despite some drawbacks, *e.g.,* the costs of the capillaries and the pre-treatment required for very complex matrices to avoid capillary clogging, it will likely be more considered in the future.

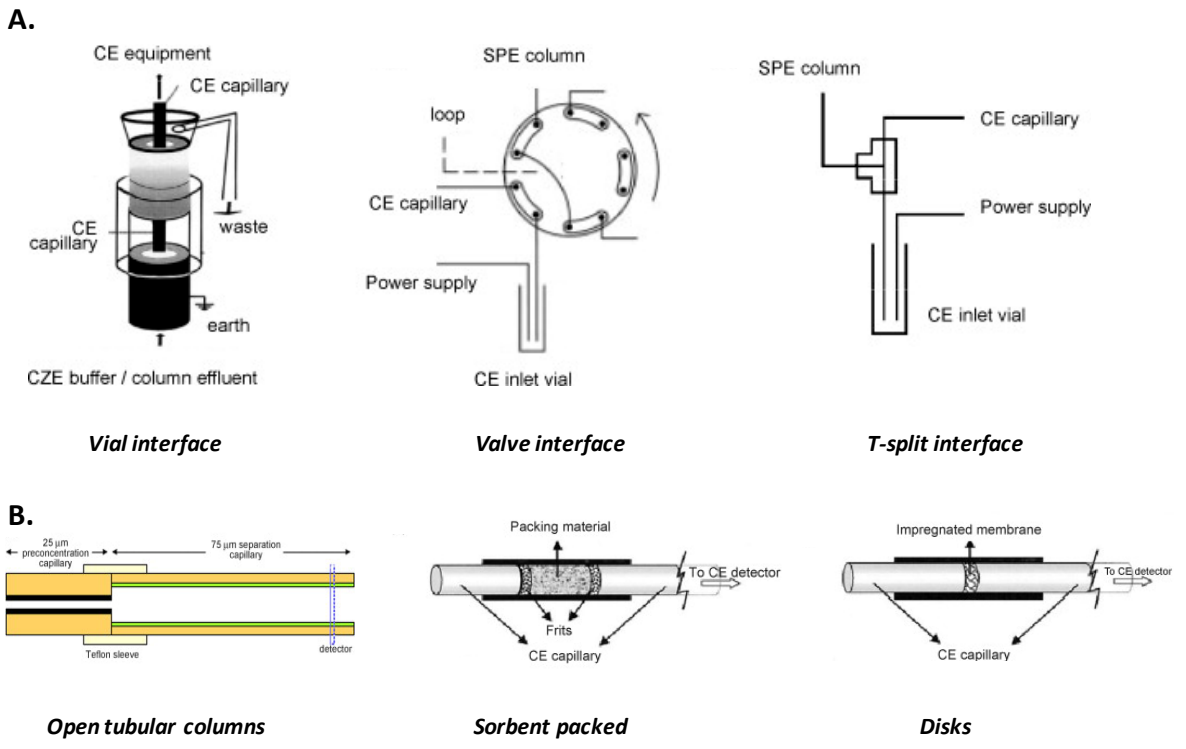

Figure 2.5. Schematic representations of automated combination of SPE with CE. A. On-line SPE-CE (vial, valve, and T-split interfaces), B. In-line SPE-CE (open-tubular columns, sorbent packed, and disks). Adapted from [18].

2.4.2 Introducing Article I

Article I presents a review of all the MEs that were off-line or in-/on-line combined with CE in the field of bioanalysis (human and animals). This review covers all the developments published from the emergence of MEs until mid-2012. Liquid-based and solid-based MEs were classified according to their principle of extraction and the improvements in their extraction performance (preconcentration factor, extraction recovery, analysis throughput, *etc.*). Two tables emphasize the studies, including the extracted analytes, the type of matrix, the sample volume, the type and volume of organic solvents, the analysis mode (separation and detection), and the preconcentration factor and/or obtained LODs. Solvent volumes ranged from *ca.* 10 nL to few mL. The highest preconcentration factors (usually *ca.* 100 but up to 10,000) were obtained with solid-based MEs.

Interestingly, this article raised the lack of existing combinations between MEs and CE-MS. Most of the studies were performed with CE-UV, which seemed to be adapted to the bioanalytical applications due to the selectivity of the extraction and the preconcentration factors obtained (LODs in the ng/mL range). However, combining the advantages of both MEs and MS detection can lead to powerful analytical strategies for clinical and forensic toxicology. Depending on the selectivity of the procedure, CE-MS combined with a sample preparation based on MEs may be applied to multi-target screening, confirmatory analysis, or compound quantitation, providing one of the greenest and least expensive analytical tools with adequate performance.

Except for in-/on-line SPE-CE, further work is mandatory to propose automated or semi-automated techniques. Currently, a large proportion of the techniques can only be off-line performed and require trained technicians.

3 Dispersive liquid-liquid microextraction

3.1 Introduction

LLE principle relies on the difference in solubility of an analyte between water (biosample) and an immiscible organic solvent leading to a selective partitioning of the targeted analyte *versus* interferents (matrix components) between the two phases. The partition coefficient K_d of the analyte is defined by its concentration C_{org} in organic phase related to its concentration C_{aq} in the aqueous phase once the system has reached the equilibrium, according to **Eq. 2.1**:

$$K_d = \frac{C_{org}}{C_{aq}} \qquad \text{(Eq. 2.1)}$$

K_d depends on the pKa value(s) of the analyte, the polarity of the solvent, and the pH of the sample which has to be typically adjusted to achieve the highest extraction recoveries. Depending on the content used to perform the extraction (*e.g.*, 15- or 50-mL polypropylene tube, or separating funnel) and the initial volume of sample (generally ≥ 1 mL), the organic solvent volume can be 10 mL or more, even up to 200 mL in separating funnels [22]. In bioanalysis, the volumes of solvents are usually comprised between 5 to 10 mL.

DLLME is a miniaturized adaptation of LLE based on a ternary component system that was introduced in 2006 by the group of Rezaee and Assadi [23]. An appropriate dispersing solvent is used to help in the dispersion of the extraction solvent into the aqueous sample. The procedure consists of a rapid injection of the solvent mixture in the sample, producing a high turbulence and the formation of tiny droplets dispersed within the sample. This turbulence is referred to as *cloudy state*. After centrifugation, the organic phase is collected and directly injected, or evaporated to dryness under a gentle steam of nitrogen and reconstituted in an appropriate solvent prior to the injection. The complete procedure is illustrated in **Fig. 2.6**. The main advantage of DLLME is the extraction time which is very short (few s) due to the extensive surface area produced by the formation of the droplets leading to an almost immediate equilibrium [23-27].

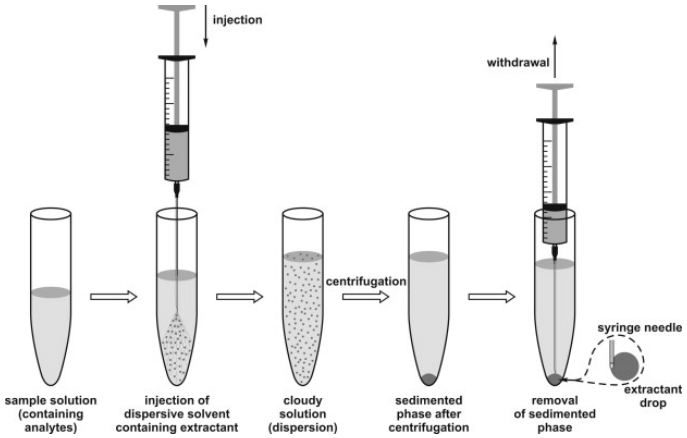

Figure 2.6. Illustration of the DLLME procedure. Reprinted from [27].

Both extraction and dispersing solvents have to be carefully selected. Typically, the extraction solvent has a low solubility in water but a higher density and high extraction capabilities. Halogenated solvents such as chloroform, dichloromethane, chlorobenzene, carbon tetrachloride, carbon disulfide, or tetrachloroethylene are frequent extraction solvents. The dispersing solvent has to be soluble in both extraction and aqueous phases, thus isopropanol, methanol, acetonitrile, or acetone are generally selected. The volume of dispersing solvent greatly affects the formation of the cloudy state, the dispersing rate of the extraction solvent in the sample, the extraction recovery, and the volume of the sedimented phase (also influenced by the volume of extraction solvent). Volumes of both solvents and sample should be thus carefully investigated. As for conventional LLE, the pH and composition of the sample clearly influence the extraction recovery, mainly in case of urine samples. It is thus important to adjust the pH of the sample prior to the extraction and take into account the conductivity of the sample [24,25].

Besides the rapid extraction time due to the rapid achievement of an equilibrium state, numerous advantages have been reported for DLLME, *i.e.*, simplicity of operation, low cost, negligible consumption of organic solvents, and high enrichment factors. DLLME has been already used in a broad range of areas, including forensic toxicology with reported applications in serum, urine, saliva, and sweat samples [25]. Many efforts have been carried out during the last years by modifying the initial methodology to further improve the simplicity of the method and expand its range of application. The evolution of DLLME is primarily based on using other dispersive and extraction solvents, as well as improving and automating the agitation step.

Although using few quantities of halogenated solvents, the latter present a high toxicity for the operator, including strong dermal irritant effects, potential carcinogenicity, degenerative cardiac diseases, nephrotoxicity and hepatotoxicity [7]. Moreover, the selection of the extraction solvent is restricted to the use of solvents having a higher density than water, leading to a tedious exhaustive collection of the sedimented phase after the centrifugation step. Therefore, two different strategies were proposed, *i.e.*, using new solvents, typically the ILs, and replacing the halogenated solvents by polar and low-density solvents [25]. ILs, defined as organic salts that remain in a liquid state at room temperature, have gained in popularity in the liquid-based MEs due to their unique properties, such as high thermal stability, negligible vapor pressure, and various viscosity [25]. Their high density and low volatility lead to the formation of stable droplets and facilitate the phase separation after the centrifugation step. They are currently considered non-toxic and green chemicals, but their toxicology remains not well studied and they probably show an environmental persistence [13]. Up to now, ILs have been mainly used for the extraction of metals.

Long-chained alcohols and hydrocarbons with lower density than water have been increasingly considered over the last years to substitute the halogenated solvents. Higher extraction recoveries for relatively polar analytes can be obtained and the organic phase is easily collected after phase separation. DLLME based on the solidification of a floating organic drop (DLLME-SFO) has been proposed to provide a simple phase collection. Indeed, using solvents with lower density and a melting point in the range of 10-30 °C allows for the collection of a solid drop at the top of the solution after an additional cooling step (ice bath). However, few solvents present these characteristics. Therefore, other strategies using low density solvents combined with home-made collection devices have been also proposed, but are not really adapted to routine analysis [25,28].

Depending on the physicochemical properties of the analytes of interest, the conventional dispersing solvents can lead to decreased extraction efficiency, especially for polar compounds which present a lower partition for the extraction solvents. Tetrahydrofuran has been proposed as an alternative, sharing the same properties than conventional dispersing solvents, but providing better recoveries. The use of surfactants as a disperser was also explored in the surfactant assisted DLLME (SA-DLLME), showing interesting advantages and absence of toxic effects. Extraction efficiencies can also be increased with the substitution of the dispersing solvent by using ultrasound to achieve ultrasound assisted emulsification-microextraction (USAEME) [29]. In this binary system, USAEME helps extend the contact of extraction solvent and sample by reversing the potential coalescence effect.

In order to adjust the density of the solvents mixture, another approach was proposed by using a quaternary system with the addition of an auxiliary solvent, generally carbon tetrachloride. The auxiliary solvent has to be of higher density than water, miscible with both extraction and dispersing organic solvents, and immiscible with the sample. The auxiliary solvent allows for the use of various extraction solvents and leads to the collection of a sedimented phase, as in conventional DLLME [30].

Although many efforts have been carried out during the last few years to achieve procedures with the highest simplicity, DLLME remains difficult to automate due to the centrifugation step. Further developments are required in this field to provide a more repeatable extraction process. The combination of DLLME with another ME (*e.g.*, SBSE or dSPE) has also provided interesting results and will be probably more considered in the future [25].

3.2 Application to the extraction of drugs of abuse

DLLME has been already successfully applied to the extraction of drugs of abuse, such as MTD [31], psychotropic drugs [32], or 7-aminoflunitrazepam [33] in various biosamples followed by LC-MS or GC-MS determination. Only few studies were performed in bioanalysis with DLLME combined to CE-UV, *i.e.*, determination of sertraline in urine [34], and extraction of MDMA, phencyclidine, and LSD in urine [35].

The potential of DLLME-CE-MS, not yet found in the literature, was evaluated in this work for the multi-target screening of numerous drugs of abuse in urine samples. Each step of the DLLME procedure was investigated with two model compounds, D-propoxyphene (D-PX) and MDMA. In order to avoid the potential matrix effects with ESI-MS detection, CE-UV configuration was used for the investigation of the procedure prior to the analysis of clinical samples with DLLME-CE-ESI-TOF/MS.

In DLLME procedure, a relatively large number of factors can have an influence on the extraction efficiency. The implementation of an experimental design methodology is thus particularly recommended to achieve the best recoveries with a small number of experiments. This strategy is also fully in accordance with the second principles of GAC which promotes the analysis of a minimum number of samples and recommends the use of chemometric tools to select the optimal experimental procedure with few experiments [6,26].

3.2.1 Design of experiments

The dispersing and extraction solvents were determined with univariate analyses due to the relative difficulty in including the nature of the solvent in a design of experiments. Among numerous combinations, dichloromethane and isopropanol gave the best results as extraction and dispersing solvents, respectively. The remaining extraction conditions were determined with the help of a screening experimental design. Seven factors X_k involved in the DLLME procedure were tested, as illustrated in **Fig. 2.7**. These factors were presumed as having a potential effect on the analytical response and were evaluated with a Plackett-Burman screening experimental design to bring out the significant factors and their positive or negative impact.

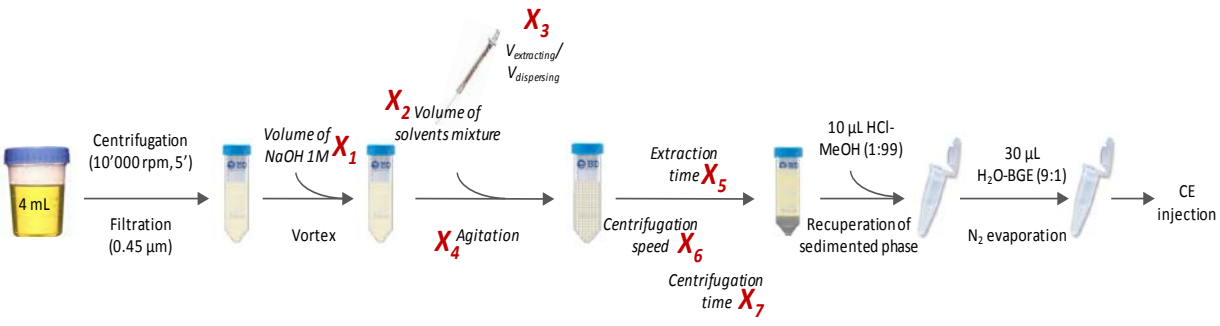

Figure 2.7. Illustration of the DLLME procedure with the experimental factors X_k tested by the experimental design.

The Plackett-Burman design was selected due to its ease of implementation, consisting in a two-level design which allows for screening a high number of factors with few experiments. The main effects were determined with a first order model as expressed by **Eq. 2.2**:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_kX_k \quad \text{(Eq. 2.2)}$$

Where Y is the response, X_k the experimental factors and b_k the coefficients. Plackett-Burman designs examine $N-1$ factors with N experiments, N being a multiple of 4. The construction of the design relies on the use of a Hadamard matrix, a square matrix which only contains -1 or +1 entries corresponding to the levels, and only exists with $N = 4, 8, 12, 16\ldots$ .The first line of the matrix is established according to the **Table 2.1**, the sign + and − representing the +1 and -1 levels, respectively.

Table 2.1. Construction of the Hadamard matrix according to the number $\boldsymbol{N}$ of experiments.

N = 8	+ + + − + − −
N = 12	+ + − + + + − − − + −
N = 16	+ + + + − + − + + − − + − − −
N = 20	+ + − − + + + + − + − + − − − − + + −
N = 24	+ + + + + − + − + + − − + + − − + − + − − − −

The following rows of the Hadamard matrix are then obtained with cyclic permutations, *i.e.*, the sign of the first factor in the second row becomes equal to the one of the last factor of the first row. This permutation is repeated until $N-2$ rows, and eventually a last N^{th} row of -1 is added. If the number of factors is lower than $N-1$, the remaining columns in the matrix are defined as *dummy factors*. Dummy factors are imaginary variables for which a change in their levels does not correspond to any physical or chemical change and is randomly or arbitrarily awarded to a given column of the design [36]. With seven factors, no dummy factors are needed and the Hadamard matrix is constructed as in **Table 2.2**.

Table 2.2. Construction of the Hadamard matrix with seven experimental factors $\boldsymbol{X_k}$

Experiment	X_1	X_2	X_3	X_4	X_5	X_6	X_7
1	+1	+1	+1	-1	+1	-1	-1
2	-1	+1	+1	+1	-1	+1	-1
3	-1	-1	+1	+1	+1	-1	+1
4	+1	-1	-1	+1	+1	+1	-1
5	-1	+1	-1	-1	+1	+1	+1
6	+1	-1	+1	-1	-1	+1	+1
7	+1	+1	-1	+1	-1	-1	+1
8	-1	-1	-1	-1	-1	-1	-1

+1 and -1 levels are determined by preliminary experiments and their values are selected to obtain the wider experimental range where a response is still observed. **Table 2.3** presents the experimental factors and their +1, 0, and -1 levels. The level 0 is used to estimate the 95% level of confidence; three rows only containing the values corresponding to the level 0 are thus randomly added to the Hadamard matrix.

Table 2.3. Experimental factors X_k and levels involved in the Plackett-Burman design.

Factors	Level		
	-1	0	+1
X_1 volume of 1 M NaOH (mL)	0.4	1.0	2.0
X_2 solvents mixture (mL)	1.0	1.5	2.0
X_3 $V_{extracting}/V_{dispersing}$ (%)	20	25	30
X_4 agitation (Y/N)[1]	N	Y	Y
X_5 extraction time (min)	0	5	10
X_6 centrifugation speed (rpm)	4000	7000	10,000
X_7 centrifugation time (min)	3	5	10

[1] Y, yes; N, No

Process efficiency (PE), defined as the absolute performance of the analytical process and discussed in the next Section, was selected as the response Y. As presented in **Table 2.4** and illustrated in **Fig. 2.8** for MDMA and D-PX, the Plackett-Burman design highlighted the factors X_2 and X_5 as having a strong effect on Y with $p \leq |0.05|$, as well as the relative importance of factors X_4, X_6, and X_7 with $|0.05| < p \leq |0.10|$.

Table 2.4. Coefficients and p-values obtained for MDMA and DPX for the experimental factors X_k

Factors	MDMA		D-PX	
	Coefficient	p-value	Coefficient	p-value
X_1 volume of 1 M NaOH (mL)	+0.021	+0.222	+0.002	+0.914
X_2 solvents mixture (mL)	+0.212	+0.024	+0.182	+0.044
X_3 $V_{extracting}/V_{dispersing}$ (%)	+0.021	+0.226	+0.023	+0.318
X_4 agitation (Y/N)	-0.046	-0.106	-0.049	-0.161
X_5 extraction time (min)	-0.156	-0.032	-0.124	-0.064
X_6 centrifugation speed (rpm)	+0.091	+0.055	+0.102	+0.078
X_7 centrifugation time (min)	+0.092	+0.054	+0.069	+0.116

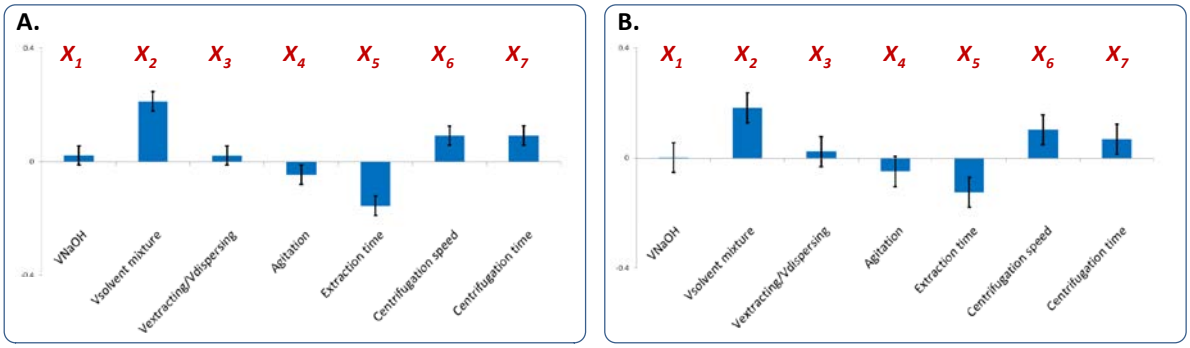

Figure 2.8. Positive and negative main effects observed for the experimental factors X_k. A. MDMA, B. D-PX. Errors bars are built with confidence interval at 95%.

Due to the relatively high number of important factors and in a perspective of keeping the consumption of organic solvents at a minimum, no additional experimental design based on optimization was performed but univariate investigations were carried out to fix the best value for each parameter. Centrifugation speed and time were found to positively affect the PE, probably due to a better phase separation after the cloudy state equilibrium. In order to keep relatively short overall extraction time, the parameter X_7 was set at 5 min, providing the best compromise between acceptable PEs, sufficient phase separation, and time. As already stated by Rezaee *et al.*, the extraction time is strongly reduced due to the immediate equilibrium provided by the large surface area or the droplets. The Placket-Burman design highlighted that letting the emulsion stand for a

time prior to the phase separation is even deleterious. This can be explained by a modification of the cloudy state over the time, leading to an equilibrium breakdown and, thus, back-extraction into the sample. Agitation of the ternary system after solvents injection was surprisingly found deleterious. The evolution of the equilibrium probably relies on a strong influence of the dispersing solvent in this ternary system. Therefore, keeping an extraction time close to zero with immediate centrifugation after solvents injection provides a significant advantage in both PE and gain of time. Finally, on the contrary of the volume ratio between both solvents, the volume of the solvent mixture was found having a strong positive effect on the response, which is explained with the probable larger surface area of the droplets, providing an enhanced equilibrium surface. However, when using a solvents volume corresponding to more than half of the sample, the cloudy state was not observed. This is probably due to the experimental conditions (proportion and nature of the selected solvents) not adapted for a stable equilibrium between the three components of the system.

3.2.2 Performance

The relevant criteria to evaluate the performance of an extraction procedure are the preconcentration factor and the analytes recoveries. The overall preconcentration obtained with a sample preparation depends on the initial volume of sample, the reconstituted volume, and the PE. In case of urine analysis, a relatively large volume is generally available, *i.e.*, up to 10 or even 50 mL. Thus, an initial volume of 4 mL of urine was used and the extraction performed with 2 mL of solvent mixture. The reconstituted volume was fixed at 30 µL, which was the minimal volume required in the vial to ensure a repeatable and reliable CE injection taking into account the equipment limitation. Assuming a theoretical PE of 100 %, an interesting 133-fold preconcentration was obtained, leading to important sensitivity enhancement. High preconcentration factors (*i.e.*, 100- to 1000-fold) are frequently observed with liquid-based MEs, which is much higher than the conventional LLE procedures. Indeed, the latter involves the use of extraction volumes based on 3:1 or even 5:1 ratios compared to the sample volume, leading to very large and unacceptable solvent quantities if the sample volume is increased. This is not the case with MEs where the sample volume can be increased while keeping the use of relatively low volumes of solvents. In the developed DLLME procedure, 0.6 mL of dichloromethane and 1.4 mL of isopropanol were used, which is considered relatively high for a “micro”-extraction, but the compromise was balanced in favor of the preconcentration by using a relatively large volume of urine to develop a highly sensitive procedure.

Combining the DLLME procedure with a CE-ESI-TOF/MS method led to LODs lower than 10 ng/mL for *ca.* thirty drugs of abuse and related compounds in spiked urine sample. As examples, amphetamines and derivatives showed LODs at 0.25-0.50 ng/mL, whereas opiates were detected at concentrations as little as 0.10 ng/mL. The performance of the DLLME-CE-ESI-TOF/MS was quantitatively evaluated for four parameters, *i.e.*, the PE, the recovery of extraction (RE), the extraction yield (EY), and the matrix effects, according to a procedure proposed by Matuszewski *et al.* [37] and revised by Marchi *et al*. [38], as illustrated in **Fig. 2.9**. As already discussed in **Chapter I**, matrix effects can be quantitatively determined by comparing the signal obtained for a matrix sample spiked after extraction to the one obtained for a neat aqueous standard. Matuszewski *et al.* also proposed a quantitative assessment of RE by comparing the signal measured with a matrix sample spiked before extraction to a matrix sample spiked after extraction. Compounds are spiked in respective samples at

a concentration which takes into account the preconcentration factor of the procedure. RE represents the performance of the extraction procedure in a given sample. Marchi *et al.* thus proposed an additional parameter, the EY, to assess the performance of the procedure for pure standards by comparing the signal measured for an aqueous solution spiked before extraction (referred to as the *neat extraction standard*) to the one of a neat standard. Finally, as previously mentioned, PE represents the performance of the complete procedure and is thus a combination of the RE and the matrix effect. PE is considered the most relevant parameter to express the overall performance of an analytical procedure and has a significant impact on the detection limits.

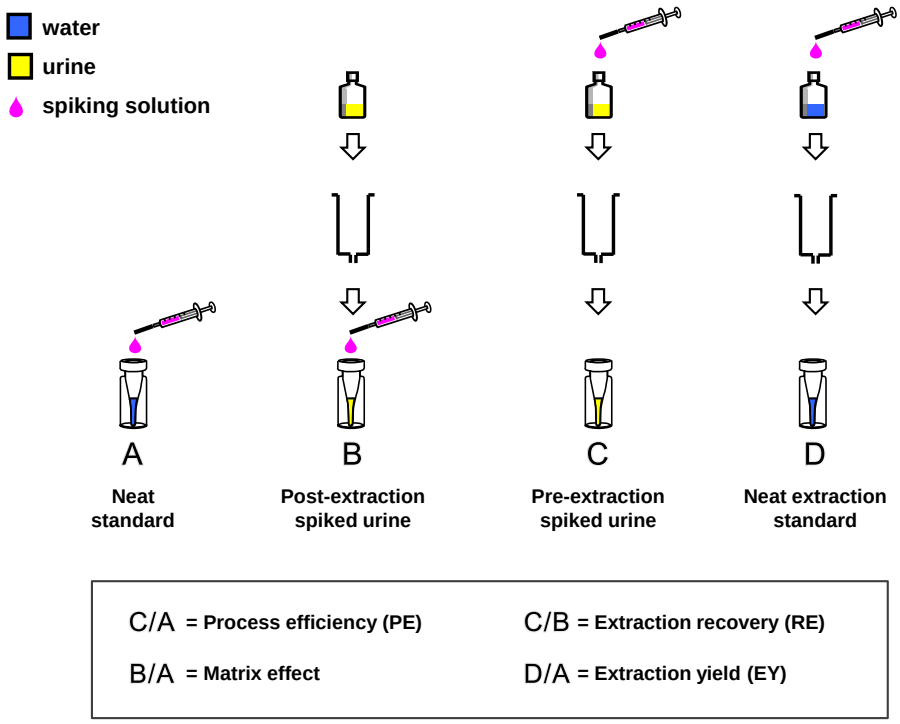

Figure 2.9. Representation of the four samples required to evaluate the process efficiency (PE), matrix effect, recovery of extraction (RE), and extraction yield (EY). adapted from [39].

A representative set of compounds of forensic interest was used for the evaluation of the performance, *i.e.,* amphetamine, MDMA, ephedrine, morphine, codeine, dextromethorphan, D-PX, and MTD. The pure performance of DLLME itself was considered satisfying with EY between 67 and 101 %, except for morphine which presents an additional acidic pKa of 9.5 and was negatively charged at the adjusted pH of the sample (> 11), and thus not extracted. 6-MAM also carries an acidic function with a pKa of 9.4 but was extracted and detected. This is probably explained by the logD value of 6-MAM at the pH of the extraction which remains higher than the one of morphine. Dextromethorphan and D-PX showed better RE than EY, likely due to the inherent salting-out effect of urine on these compounds. Both compounds as well as MTD also presented a significant ion suppression (more than 50 %) which means that the selectivity of the DLLME procedure against the matrix interferents was not sufficient for them. Finally, except for morphine, PEs were between 32 and 76 %, which is acceptable taking into account the high preconcentration factor of the procedure.

The values obtained for PEs highlight the challenges encountered with the development of sample preparation procedures for screening purpose, *i.e.,* to find the best compromise between a generic procedure and a sufficient sample clean-up. This is particularly the case for drugs of abuse which

present various physico-chemical properties leading to strong differences in their extraction behavior. Therefore, it is important to couple the DLLME with a sensitive detection to improve the overall performance. For example, a PE of 32 % was obtained for MTD with the proposed DLLME-CE-ESI-TOF/MS procedure, but a LOD of 0.25 ng/mL was estimated, which is sufficient for the detection of MTD in screening approaches.

A drawback of the DLLME was noted with a rather poor repeatability of the extraction itself. The number of extraction step that have to be manually performed is quite high, leading to additive variances during the procedure. Mainly, it is difficult to be repeatable during the injection of the solvents mixture into the sample and during the sedimented phase collection, which was also found to provide each time a slightly different volume. Both steps shall be improved with the automation of the procedure. Currently, as recommended for the analytical procedures involving a sample preparation, the IS correction shall be used to enhance the low repeatability of the extraction process.

3.3 Introducing Article II

Article II presents the development of a DLLME procedure coupled to CE-ESI-TOF/MS for the screening of drugs of abuse in urine samples. The article focuses on the optimization of the extraction procedure, aiming at providing the highest PEs and preconcentration factor. The operating conditions of the CE-ESI-TOF/MS method were also investigated to have a sufficient selectivity and resolution between the compounds and the highest sensitivity. Using a BGE composed of 25 mM ammonium formate at pH 2.5 allowed for the separation of the compounds in less than 15 min. Isobaric compounds such as MDEA and MBDB or ephedrine and pseudo-ephedrine were almost baseline resolved with these conditions. Compounds sharing similar migration time were separated according to their different *m*/*z*. **Table 2.5** summarizes the main performance obtained with the developed DLLME procedure in terms of preconcentration factor, solvents amounts, and whole extraction time (including agitation and evaporation steps), and compared to the conventional performance observed with LLE.

Table 2.5. Performance obtained for DLLME compared to conventional LLE.

Parameters	DLLME	LLE
Preconcentration factor	133	10-100
Volume of solvents	0.5 mL *per* mL of sample	*ca.*3 mL *per* mL of sample
Extraction time	< 10 min *per* sample	*ca.* 30-60 min *per* sample

The complete procedure was also used for the analysis of two clinical samples. Compounds identification was performed with accurate mass, comparison of migration times with standard solutions, and detection of specific metabolites. In the first one, COC and two metabolites, cocaethylene and AEME, were detected. These two metabolites gave additional information, first a co-consumption of COC and ethanol, which leads to the formation of cocaethylene (*trans*-esterification of COC by ethanol), and secondly the route of administration, AEME being a pyrolysis product only detected in case of smoked COC. In the second sample, MDMA and its metabolite MDA were detected.

A French version of **Article II** is presented in **Appendix II**.

4 Disposable pipette extraction

4.1 Principle

SPE involves the extraction of compounds based on their partitioning between a solid phase (sorbent) packed within two fritted disks in a 1-mL, 3-mL, or 6-mL polypropylene cartridge, and a liquid phase (sample and washing/elution solutions). Conventional sorbents are composed of irregular-shaped rigid particles of usually 40- to 60-µm diameter composing a fully porous material. A large variety of silica-based or polymer-based sorbents can be used depending on the physico-chemical properties of the targeted analyte(s). Silica-based sorbents are manufactured by binding functional groups, such as C_{18}, C_8, C_6, C_4, C_2, phenyl, cyanopropyl, diol, or propyl carboxylic acid to activated silica. Due to steric factors, a large proportion of residual silanol species remain after the manufacture, leading to secondary interactions during the extraction, mainly for C_2 and C_4 phases. These secondary interactions can be advantageous to retain compounds presenting both hydrophobic and ionic properties; otherwise silica-based sorbents with endcapped residual silanols are also commercially available. Polymer-based sorbents are mostly composed of polystyrene-divinylbenzene or divinylbenzene-*N*-vinylpyrrolidone resins modified with polar or ionic groups. Polymer-based sorbents present a high stability at extreme pH values and can be dried without deleterious effect on the recovery, on the contrary of silica-based sorbents [40].

The extraction process relies on polar, non-polar, or ionic interactions between the sorbent and the analytes. Non-polar sorbents (*e.g.,* C_{18}, C_8, phenyl, *etc.*) are widely used to extract non-polar analytes from aqueous sample. Careful selection of the elution solution is required to have a selective extraction of analytes *versus* contaminants. Polar sorbents (*e.g.*, diol, aminopropyl, *etc.*) involve dipole-dipole interactions or hydrogen-bonding with polar groups of the analytes. If a polar sorbent is selected, an initial LLE step is recommended prior to SPE for aqueous samples. Otherwise, when an aqueous solution is loaded, the analytes will not be retained by the sorbent and will be directly eluted due to the polarity of the sample. The analytes transfer into a less polar solvent is thus required. Ionic interactions are achieved using cation-exchange or anion-exchange sorbents. In cation-exchange SPE, analytes under their cationic form are retained with electrostatic interactions to anionic surface groups. Elution of the analytes is performed (i) with modification of the pH (neutralization of the analytes or the sorbent), (ii) by increasing the ionic strength, and/or (iii) by using a buffer with high content of cationic species showing a higher affinity for the sorbent. In case of samples with high salts content such as urine, an initial dilution step is recommended. Sorbents with carboxylic acid groups are referred to as *weak cation-exchanger* (pH-dependant ionization) and sulfonic acid groups as *strong cation-exchanger* (ionized over the whole pH range). Anion-exchange SPE exhibits the same properties and retains compounds under their anionic state. The most common sorbents have amine groups on their surface [41,42].

In bioanalysis, mixed-mode sorbents are frequently used and show a higher selectivity compared to the matrix while broadening the range of compounds extraction. Mixed-mode sorbents are typically composed of hydrophobic chains or groups combined with an ion-exchange functional group. Using mixed-mode sorbents and modifying the pH conditions during the extraction allow for a sequential retention of basic and acidic compounds *via* hydrophobic and ionic interactions, leading to two different eluates that can be gathered prior to the evaporation and injection.

Besides silica- and polymer-based sorbent, other supports are commercially available, including Florisil© (magnesium silicate), surfactant-modified sorbents, molecular recognition sorbents such as MIPs, immunosorbents, or aptamers; RAM, and monoliths [43].

MIPs are stable polymers with molecular recognition abilities which thus provide a high extraction selectivity. Synthetic materials with artificially generated recognition sites are used which specifically retain a target molecule in preference to other closely related compounds. The materials are obtained by polymerizing functional and cross-linking monomers (*e.g.*, methacrylic acid, ethylene glycol dimethacrylate) around a template molecule. Once the polymerization is achieved, the template molecule is removed by extensive washing step and binding sites (cavities) that present complementary size, shape, and functionalities to the target analyte are established. MIPs provide the same selective retention than immunosorbents without any limitations in stability. Molecularly imprinted solid-phase extraction is performed by using a small amount (50-500 mg) of MIPs packed into the cartridges. Specific interactions are maximized in a low-polarity solvent, thus an initial LLE step can be preliminary carried out for aqueous samples. If biosamples are directly loaded, MIPs behave like a reverse-phase sorbent. Non-specific interactions will thus retain both analyte and matrix components. In order to reduce these hydrophobic interactions, polymers with hydrophilic surface properties can be used for synthesis of water-compatible MIPs [44,45]. Immunosorbents, also referred to as immunoaffinity extraction, involve the same molecular recognition and are obtained by linking monoclonal or polyclonal Abs to a solid support packed in the cartridge. In immunoaffinity extraction, a careful attention has to be paid on numerous parameters, including Ab selection, extraction conditions, possible cross-reactivity, and storage conditions [46]. Aptamers are short, single-stranded, and synthetic oligonucleotides that fold in a shape capable of binding to a target analyte with high specificity. Although providing some advantages such as the few amounts of the target molecule required as template, their use is still limited [43].

SPEs performed with polypropylene cartridges involve the use of *ca.* 1-5 mL of organic solvent for each extraction. This volume can be reduced by using disks or well-plate format, and significantly decreased or eliminated with solid-based MEs. MEs such as SBSE and SPME only require few amounts of solvents for analyte desorption; however, although providing numerous advantages, these procedures are not quantitative, *i.e.*, an exhaustive analyte extraction is not achieved due to the retention principle based on the distribution equilibrium between the sample and the sorbent phase. This is not the case with disposable pipette extraction (DPX).

DPX belongs to the *dispersive solid-phase extractions* (dSPE) where the extraction is performed by adding the sorbent phase in a powder form to the sample. The most popular dSPE is the QuEChERS method, which stands for *Quick, Easy, Cheap, Effective, Rugged, and Safe*, and is widely used in food analysis [47]. DPX was developed and patented by Prof. William E. Brewer (University of South California, USA, Owner-President of DPX Labs, Columbia, USA) in 2007. DPX consists of a modified standard 1- or 5-mL pipette tip which contains a loosely SPE sorbent free to disperse [48]. Sample and solvents can flow in and out of the tip through this sorbent. Two frits are contained in the modified pipette tip to retain the sorbent, one on the lower end of the tip through which solutions can flow and one at the upper end of the tip which avoid the contamination of the pipette/syringe by solvents. On the contrary to SPE, where the analytes get in contact with the sorbent particles only once, in DPX the sample is mixed with the loosely material and every particle faces the analytes

several times [47,49,50]. This provides a fast and efficient extraction. As for traditional cartridges, pipette tips with numerous sorbents are commercially available, including reverse-phase polystyrene-dinvinylbenzene copolymer, strong cation exchanger, weak cation exchanger, graphitized carbon, or QuEChERS. A full automation of the extraction process is possible using a Multi Purpose Sampler provided by GERSTEL GmbH & Co (Mülheim an der Ruhr, Germany), allowing for an extraction process of less than 3 minutes *per* sample.

DPX has seen a growing interest in bioanalysis due to multiple advantages, such as the minimal elution volumes, the high REs obtained for the compounds, the limited volumes of sample required, the lower costs, the possibility of automation, and the ease of use. It has been already successfully used for the extraction of drugs of abuse in urine [48], THC and nor-THC in whole blood and urine [51], opiates in vitreous humor [52], and biperiden and antipsychotic drugs in urine [53]. The studies today remain quite limited but DPX will likely see a significant increase in its use the following years.

The potential and performance of DPX combined with CE analysis were evaluated during this thesis work for the extraction of both basic and acidic compounds of forensic or clinical interest in urine samples.

4.2 Application to screening analysis

A mixed-mode sorbent was selected to allow for a sequential extraction of basic and acidic compounds. DPX-CX 1-mL pipette tips are composed of 20-mg copolymer styrene divinylbenzene sorbent with sulfonic acid groups retaining acidic compounds by hydrophobic interactions with the copolymer and basic analytes *via* ionic interactions with the sulfonic acid groups. The purchased DPX-CX pipette tips contained a transport adaptor for GERSTEL Multi Purpose Sampler automation. However, a free loan of the autosampler at this time was not possible and the loan prices prohibitive. A manual procedure was thus developed using a 10-mL plastic syringe (Glass Technology, Switzerland) and a BD Microlance 3 needle (Milian SA, Switzerland) of 24G × 1'' dimensions, as shown **in Fig. 2.10**.

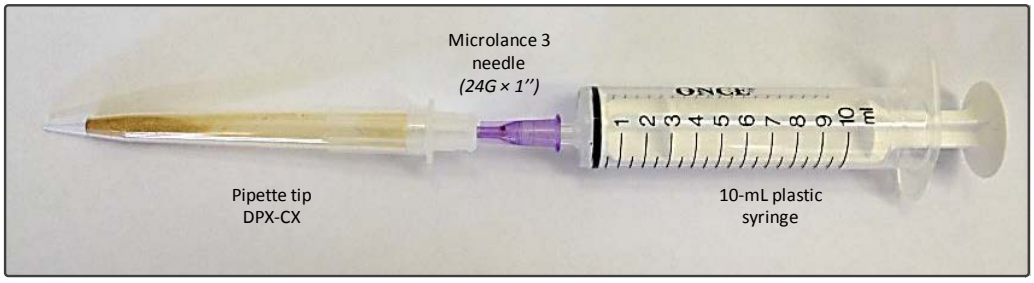

Figure 2.10. Manual adaptation for the use of DPX-CX pipette tips with transport adaptor.

The typical steps of a DPX procedure are depicted in **Fig. 2.11** and involve the sorbent condition by aspiration of the appropriate solvent to activate the functional sites, the aspiration of the sample, the mixing with air (equilibration time) for a selected time, the sample discharge, the washing steps, and the (sequential) elution of the compounds by addition of the solvent from the top of the tip followed by multiple aspirations with air to ensure a complete desorption of analytes. The extract is then directly injected or evaporated prior to reconstitution.

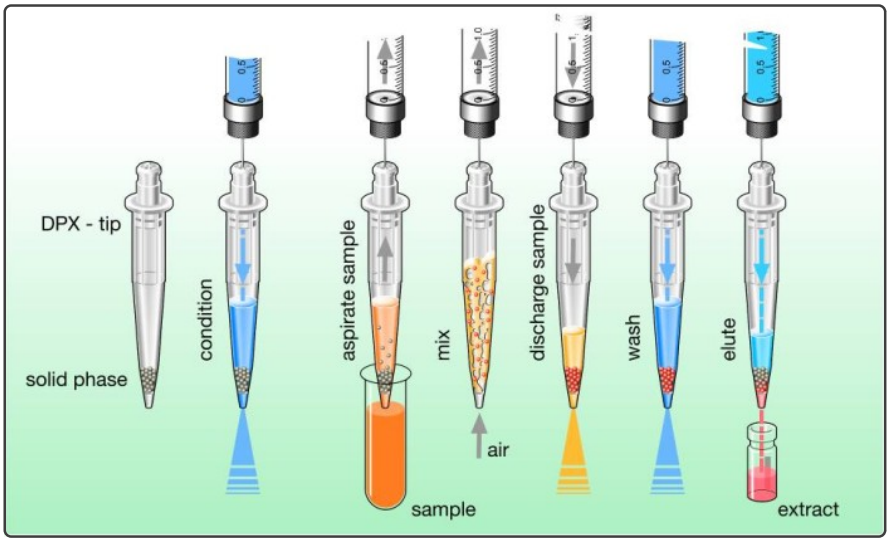

Figure 2.11. Representation of the typical steps involved in a DPX procedure. Courtesy of GERSTEL Inc., Linthicum (MD, USA).

The procedure was investigated with a set of acidic (salicylic acid, diclofenac, phenobarbital, and warfarin) and basic (fentanyl, ketamine, MDMA, and D-PX) model compounds. As in **Section 3.2**, CE-UV was used for the method development to avoid any matrix effects encountered with ESI-MS detection. A BGE composed of 20 mM ammonium acetate at pH 9.0 was used for the analysis of acidic elution fraction, while a BGE consisting of 20 mM ammonium formate at pH 2.5 was selected for the analysis of the basic fraction. The selection of the extraction conditions was performed with univariate experiments due to the high variability expected with the manual extraction. EY was monitored during the method development to assess the performance of the DPX procedure itself; the selected conditions were then confirmed with the evaluation of the obtained PEs in urine samples.

The developed procedure consisted in the following steps. The sorbent was first conditioned with 700 µL of methanol, 500 µL of water, and 500 µL of 150 mM HCl. Five hundred milliliters of the sample were mixed with 200 µL of 150 mM HCl and aspirated into the tip. Acidification of the sample was required to maximize the ionic interactions for basic compounds with the support as well as hydrophobic retention of acidic compounds. With 200 µL of 150 mM HCl, the pH values measured for different urine samples were all below 1.8, which was sufficiently low for a large variety of toxicological compounds. After the sample loading, the solution was mixed with air for 1 min prior to flushing out the matrix at a rate of two drops/s. Five hundred microliters of a 0.5-% formic acid solution were used to wash the sorbent. Elution of the fraction containing the acidic compounds was performed with 700 µL of methanol, followed by the elution of basic compounds with 700 µL of a mixture composed of acetonitrile:dichloromethane:ammonia 75:20:5 (*v/v/v*). Both factions were evaporated to dryness and each reconstituted in 25 µL of water prior to the CE injection.

Although the pipette tips are designed to contain up to 1 mL of solution, each step was performed with maximum 700 µL to still have a sufficient void volume for air mixing. A compromise between extraction time, solvents amounts, and EY was found for each step. Enhancing the volumes of solution or repeating the steps were not found to significantly increase the extraction efficiency while enhancing the variability and time due to the additional manipulations.

For acidic compounds, EY between 85 and 122 % were obtained (*N* = 6). However, the developed procedure was not adapted to urine samples with CE-UV analysis due to (i) an insufficient matrix clean-up and (ii) an insufficient salts removal, which led to unstable electrophoretic processes. This effect can also be observed with conventional SPE and is inherent to the CE analysis. Therefore, further investigations are required to allow for the extraction and analysis of acidic compounds. Regarding the extraction of basic compounds (*i.e.*, fentanyl, ketamine, MDMA, and D-PX), acceptable EYs between 43 and 88 % were obtained. The lower EY was observed for MDMA, probably explained by compound loss during the evaporation step. This effect is well known for semi-volatile compounds such as amphetamines and does not depend on the performance of the extraction procedure itself. Attempts in lowering this possible evaporation by acidification prior to the evaporation were not successful. PEs were between 31 and 78 %. Urine matrix did thus not influence the extraction of the selected compounds with close values obtained for EY and PE. Repeatability of the procedure was evaluated for 6 consecutive standard extractions with correction of the CE injection variability by adding an internal standard (lidocaine). RSDs on peak areas were lower than 13 % for basic compounds, which was found acceptable for a completely manual extraction procedure. For acidic compounds, RSDs up to 45 % were obtained, which confirm the need for additional investigations.

The performance of the developed DPX method was compared to a typical SPE procedure using Oasis© MCX cartridges (Waters, Milford, USA) which share similar sorbent properties than DPX-CX. An optimized SPE procedure from the literature for the same classes of compounds was applied [54]. For most of the acidic and basic compounds, EYs were more than 2-fold higher for the developed DPX-CX procedure than for SPE-MCX, as exposed in **Table 2.6** which summarizes the main performance obtained for the developed DPX procedure. The comparison with SPE illustrates the intrinsic properties of the DPX pipette tips with enhanced surface and time contact between the analytes and the loosely sorbent, leading to higher extraction efficiencies. However, besides the higher efficiencies observed for the targeted analytes, higher extraction of the matrix components was also observed. Indeed, CE-ESI-TOF/MS analysis of the basic eluate led to PEs between 14 and 32 %, explained by substantial ion suppression due to matrix effects. The sample clean-up was thus not fully optimal and washing steps should be further investigated. The complete DPX-CE-ESI-TOF/MS procedure was applied to two forensic urine samples, providing a sufficient sensitivity with the detection of MTD and its specific metabolite EDDP in the first sample, while COC, norCOC, cocaethylene as well as MTD and EDDP were detected in the second sample.

Table 2.6. Performance obtained for DPX compared to SPE.

Parameters	DPX	SPE
Preconcentration factor	20	20
Volume of solvents	4.2 mL *per* mL of sample	2.2 mL *per* mL of sample
Extraction time	< 10 min *per* sample	< 10 min *per* sample
EY	43-88 %	40-43 %

These preliminary results are thus promising for the use of DPX in bioanalysis. Up to now, only LC and GC have been used in combination with DPX, allowing for a complete automation of the analysis. This is currently not possible with conventional CE instruments. DPX presents interesting advantages and numerous sorbents are already available. However, after few years of commercialization, limited studies have been published until today. Possible hypotheses are the costs generated by the pipette

tips (*ca.* € 5.-) and the dedicated commercial autosampler, as well as the relatively high solvent consumption for a miniaturized technique, explained by the amount of sorbent in the tip, *i.e.*, 20 mg.

5 Conclusions

Since the first attempts more than two decades ago in miniaturizing the sample preparation step, numerous developments were performed with varying degrees of success. Some of them are today widely used and commercially supported. A significant decrease of solvents consumption has been observed, which is completely in agreement with the current sustainable concern. Not only reduced solvents amounts have been obtained, but also lower volumes of sample, higher extraction efficiencies, higher preconcentration factors, and higher throughputs with semi- or full automation and on-line coupling to chromatographic methods.

The potential of the combination of MEs with CE has been shown in this Chapter with the development of a liquid-based ME for the extraction of a wide range of drugs of abuse prior to CE-ESI-TOF/MS analysis, as well as the preliminary results obtained with DPX, a solid-based ME which will undoubtedly see its use increased in bioanalysis.

Numerous adaptations of existing MEs are published every year, especially for liquid-based MEs, but are not really adapted for a daily use in clinical or forensic laboratories. Some relevant trends or innovations in MEs seem to emerge, such as the use of ILs instead of organic solvents in numerous liquid-based MEs. Nevertheless, despite the promising results already observed in terms of performance, some toxicological studies are still required to ensure their lack of toxicity for human health and the environment. DBS, due to the ease of collection (finger pick) and the possibility of storage at room temperature, will probably be increasingly considered, mainly for clinical purposes such as TDM. One other promising approach in clinical and forensic toxicology is the implementation of *in vivo* SPME sampling for blood analysis. SPME involves the extraction of analytes by exposing an extracting phase bound to a fiber to the sample for a defined period of time. The number of moles n of analytes extracted follows the **Eq. 2.3**:

$$n = K_{fs} \times V_f \times C_0 \quad \text{(Eq. 2.3)}$$

With K_{fs} the distribution coefficient of the analyte between the extracting phase and the sample, V_f the extracting phase volume, and C_0 the initial concentration of a given analyte in the sample [55]. According to **Eq. 2.3**, the amount of analyte extracted does not depend on the volume of the sample, which point out the usefulness of SPME for *in vivo* sampling with direct exposure of the probe to the circulating blood. As an example, an *in vivo* SPME procedure has been proposed to monitor and quantify intravenous concentrations of drugs and metabolites in living animals [56]. A sterile SPME probe was tested in animals with insertion into the peripheral vein through a standard medical catheter and the extraction was performed in less than 5 min. Biocompatible SPME probes are already available with sorbents coated on a fine-gauge surgical steel wire which does not cause an immune response in experimental animals. Confirmation of the potential of *in vivo* SPME sampling is still required to ensure its full applicability for drug monitoring in humans.

6 References

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7 Scientific publications

Article I

Microextraction techniques combined with capillary electrophoresis in bioanalysis

I. Kohler, J. Schappler, S. Rudaz, Anal Bioanal Chem 405 (2013) 152

Article II

Dispersive liquid-liquid microextraction combined with capillary electrophoresis and time-of-flight mass spectrometry for urine analysis

I. Kohler, J. Schappler, T. Sierro, S. Rudaz, J Pharm Biomed Anal 73 (2013) 82

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REVIEW

Microextraction techniques combined with capillary electrophoresis in bioanalysis

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Abstract Over the past two decades, many environmentally sustainable sample-preparation techniques have been proposed, with the objective of reducing the use of toxic organic solvents or substituting these with environmentally friendly alternatives. Microextraction techniques (MEs), in which only a small amount of organic solvent is used, have several advantages, including reduced sample volume, analysis time, and operating costs. Thus, MEs are well adapted in bioanalysis, in which sample preparation is mandatory because of the complexity of a sample that is available in small quantities (mL or even μL only). Capillary electrophoresis (CE) is a powerful and efficient separation technique in which no organic solvents are required for analysis. Combination of CE with MEs is regarded as a very attractive environmentally sustainable analytical tool, and numerous applications have been reported over the last few decades for bioanalysis of low-molecular-weight compounds or for peptide analysis. In this paper we review the use of MEs combined with CE in bioanalysis. The review is divided into two sections: liquid and solid-based MEs. A brief practical and theoretical description of each ME is given, and the techniques are illustrated by relevant applications.

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Abbreviations

μ-SLM	Micro-supported liquid membrane
BGE	Background electrolyte
C^4D	Capacitively-coupled contactless conductivity detector
CB-ICE	Chip-based immunoaffinity capillary electrophoresis
CE	Capillary electrophoresis
CME	Centrifuge microextraction
CM-LPME	Carrier-mediated liquid-phase microextraction
CM-SDME	Carrier-mediated single-drop microextraction
CZE	Capillary zone electrophoresis
DEHP	bis(2-Ethylhexyl) phosphate
DI-SPME	Direct-immersion solid-phase microextraction
DLLME	Dispersive liquid–liquid microextraction
DMD-LPME	Droplet–membrane–droplet liquid-phase microextraction
DSDME	Directly suspended droplet microextraction
EK	Electrokinetic injection
EME	Electro membrane extraction
ENB	1-Ethyl-2-nitrobenzene
ESI	Electrospray ionization
FASI	Field-amplified sample injection
GC	Gas chromatography
HS	Headspace
i-PrOH	Isopropanol
ILBE	In-line back-extraction
IT	Ion trap
LC	Liquid chromatography
LIF	Laser-induced fluorescence

LLE	Liquid–liquid extraction
LLLME	Liquid–liquid–liquid microextraction
LOD	Limit of detection
LVSS	Large-volume sample stacking
MCE	Microchip capillary electrophoresis
ME	Microextraction
MeCN	Acetonitrile
MEKC	Micellar electrokinetic chromatography
MeOH	Methanol
MEPS	Microextraction by packed sorbent
MIP	Molecularly imprinted polymer
NACE	Non-aqueous capillary electrophoresis
NPOE	2-Nitrophenyl octyl ether
NSAIDs	Non-steroidal anti-inflammatory drugs
OLBE	On-line back-extraction
PAH	Polycyclic aromatic hydrocarbons
PF	Preconcentration factor
PP	Protein precipitation
RAM	Restricted-access material
SBSE	Stir-bar-sorptive extraction
SL	Sheath liquid
SPE	Solid-phase extraction
THF	Tetrahydrofuran
tITP	Transient isotachophoresis
TOF	Time-of-flight
UHPLC	Ultra-high-pressure liquid chromatography
USAEME	Ultrasound-assisted emulsification microextraction

Introduction

The overall analytical procedure includes several consecutive steps—sampling, sample storage, sample preparation, separation of target analytes, detection, and data treatment. For analysis of complex samples and matrices, for example biological, environmental, or food analysis, the sample preparation is of utmost importance for obtaining the analytes of interest in a suitable injection solution able to provide reliable and accurate results. Sample preparation has substantial objectives before sample injection, including:

1. reducing or eliminating matrix interferents or undesired endogenous compounds;
2. increasing selectivity for targeted analyte(s);
3. preconcentrating the sample to enhance sensitivity; and
4. stabilizing the sample by reconstituting it in an inert solvent.

Although great improvements have been made in the development of fast separation techniques, sample pretreatment remains the most time-consuming step, accounting for ca two thirds of the entire analytical procedure [1]. In addition, because of the lack of automation of several offline procedures, sample preparation is also regarded as a primary source of analytical errors that can significantly affect the throughput [2].

Sample preparation can be based either on selective methods, e.g., the widely used solid-phase extraction (SPE) and liquid–liquid extraction (LLE), or non-selective methods, e.g., using membrane techniques or protein precipitation (PP). A common feature of all these conventional sample-preparation techniques is the relatively high consumption of solvents that are environmentally hazardous and health risks for humans. The advent of the concept of "green chemistry" at the beginning of the 1990s emphasized the need for non-toxic and environmentally friendly analytical procedures. The concept also promoted the use of environmentally sustainable sample-preparation methods with the development of solvent-free or miniaturized extraction methods [3, 4]. Different approaches can be envisaged when developing environmentally sustainable sample preparation:

1. solventless procedures [5, 6];
2. substitution of organic solvents with less-toxic alternatives, for example use of supercritical-fluid extraction, cloud-point extraction, subcritical water extraction, or extraction with ionic liquids [7, 8]; or
3. use of microextraction techniques (MEs), in which miniaturization of the extraction procedure not only minimizes the use of organic solvents but also the sample volume required.

MEs are defined as non-exhaustive procedures that use very small volumes of the extracting phase and for which the volume of sample is relatively large compared with that of the extracting phase [9]. MEs reduce or eliminate the consumption of solvents while simultaneously reducing sample volume, analysis time, and operating costs [10]. Many techniques have been developed over the last few decades for a variety of applications, i.e., in environmental analysis (pesticides, hormones) [11–13], food analysis [11, 13–15], and bioanalysis [16, 17] for clinical, toxicological and forensic purposes [18, 19] or doping analysis [20]. In bioanalysis, often only small amounts of the sample are available, typically in the mL range for urine and in the μL range for serum or plasma or alternative matrices, for example sweat, saliva, or tears. Because of the complexity of theses matrices and the low concentrations of the target analytes compared with endogenous interferents, sample preparation is mandatory, and MEs are particularly well adapted for this purpose.

A variety of analytical techniques, including separation-based approaches, can be implemented in combination with MEs in bioanalysis. Non-polar and volatile compounds are conveniently analyzed by gas chromatography (GC), whereas liquid chromatography (LC), including ultra-high-

pressure liquid chromatography (UHPLC), is extensively used in bioanalysis for both quantitative and qualitative purposes, because of its wide applicability to a large number of compounds with different physicochemical properties. Capillary electrophoresis (CE) is another powerful separation technique that is often used in bioanalysis, because of its high separation efficiency. As very small amounts of (μL range) or no organic solvents are required for CE analysis, its use in combination with ME techniques is regarded an attractive, environmentally sustainable analytical tool. Extracts can be directly injected for analysis, or evaporated and reconstituted in a very small volume. Because a few nL of sample is injected in CE, very high preconcentration factors (PFs) can be achieved, enhancing the overall sensitivity, which is a disadvantage of the capillary format.

Applications of ME techniques before to CE analysis have been reported over the past few decades in bioanalysis of low-molecular-weight compounds or small peptides. In this paper we review the MEs used in bioanalysis and combined with CE. It is divided into two sections: liquid and solid-based MEs. MEs are classified according to their extraction principle and improvement of extraction performance. A brief description and the theoretical concepts of each ME technique are introduced and discussed, and illustrated by relevant applications.

Liquid-based microextraction techniques

LLE, which involves partition of analytes between an aqueous sample and water-immiscible organic solvent, has been widely used in bioanalysis because of its simplicity and ease of implementation. LLE suffers from major drawbacks, for example emulsion formation at the interface of the immiscible phases, lack of selectivity (co-extraction of endogenous interferents), lack of automation, and use of large sample volumes and large amounts of toxic organic solvents that are environmentally harmful (up to 10 mL per mL of sample) [10, 16, 17, 21].

New methods based on the LLE principle or with original set-ups have been developed during the last two decades to overcome these drawbacks. Miniaturization of LLE has led to several new liquid-based ME techniques in which the total volume of organic solvent required has been reduced to the *sub*-mL level.

In 1996, Cantwell and co-workers [22] and Dasgupta and co-workers [23] were the first to propose the use of a solvent drop in the μL range as extractant, laying the foundation for liquid-phase microextraction (LPME). Cantwell and co-workers used an 8-μL drop of *n*-octane held at the end of a Teflon rod to extract 4-methylacetone from water [22], whereas Dasgupta and co-workers extracted sodium dodecyl sulfate (SDS) from a water sample with only 1.3 μL chloroform [23]. A variety of liquid MEs based on LPME were subsequently developed, leading to a large selection of miniaturized techniques that are still evolving. A schematic diagram of these techniques, based on their principle of extraction, is given in Fig. 1. All of the bioanalytical applications that use LPME-based techniques before CE are listed in Table 1.

Liquid-based ME techniques are derived either from single-drop microextraction (SDME), in which a single drop of water-immiscible solvent suspended from the tip of a syringe is immersed in the aqueous sample, or hollow-fiber liquid-phase microextraction (HF-LPME), in which a hollow polymeric fiber is used as a support for the acceptor (aqueous or organic) phase.

Single-drop microextraction (SDME)

SDME was introduced in 1997 by Jeannot et al. and He et al. [24, 25]. In the first study, a 1-μL drop of *n*-octane was suspended in a stirred aqueous sample from the tip of a microsyringe needle. After a few minutes, the drop was retracted into the needle and injected directly for gas chromatographic (GC) analysis [24]. He and Lee used the same method with a 1-μL drop of toluene that was immersed in the aqueous sample for 15 min before retraction and injection [25]. SDME uses very small amounts of organic extraction solvents, which enables important PFs to be achieved. The main problems with this method are lack of droplet stability at high stirring speeds and the high manual dexterity required. Moreover, SDME is only suitable for relatively non-polar analytes and suffers from low recovery and repeatability. Therefore, SMDE was regarded as be not suitable for biological matrices, in which an extra filtration step is necessary [2, 11, 26]. Many derived techniques based on SDME were thus proposed (Fig. 1), including liquid–liquid–liquid microextraction (LLLME) or dispersive liquid–liquid microextraction (DLLME), and used in combination with CE to obtain sufficient selectivity, sensitivity, and repeatability in bioanalysis.

Liquid-liquid-liquid microextraction (LLLME)

LLLME, also referred to as LPME by back-extraction, was first introduced in 1998 by Ma and Cantwell [27] and is particularly suitable for water-soluble analytes, for example ionizable compounds. In LLLME, the targeted analytes are first extracted from the aqueous sample (donor) into a water-immiscible organic phase (acceptor I) and then back-extracted into a separate aqueous phase (acceptor II). The transfer occurs by manipulating the pH in the donor and acceptor phases. LLLME is particularly suitable for CE analysis, because of the direct injection of the aqueous acceptor phase into the system.

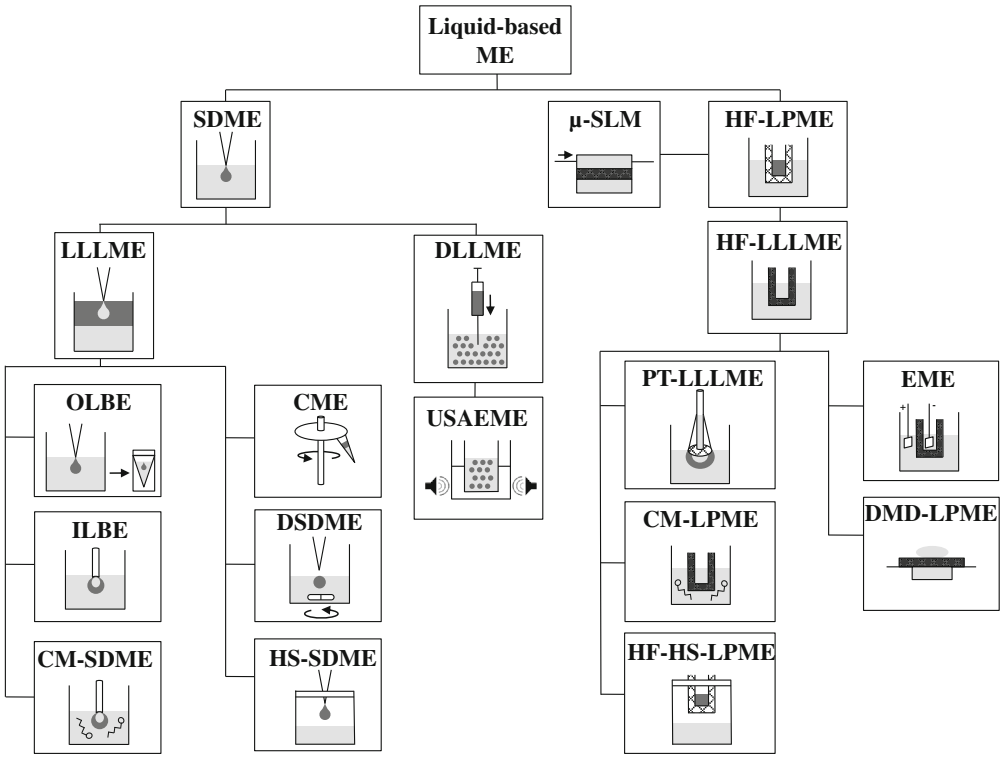

Fig. 1 Classification of liquid-based microextractions used in combination with CE. *Light gray*, aqueous phase; *dark gray*, organic phase; *cross hatched*, membrane or fiber

Extraction improvement

The particular configuration of CE enables on-line or in-line back-extraction to be performed. On-line back-extraction (OLBE) with field-amplified sample injection (FASI) was developed for analysis of cocaine and thebaine in urine samples [28]. Eight milliliters of urine were placed in a vial, and a 2-μL drop of chloroform was generated at the tip of a syringe and immersed in the sample. After extraction for 5 min, with stirring, the chloroform drop was retracted and transferred to another vial that was sealed with 40 μL acidified water for back-extraction. OLBE was performed by carefully immersing the capillary tip in the water plug. During high-voltage application, FASI occurred, and charged analytes moved rapidly from the organic phase to the capillary, stacked at the boundary with the high-conductivity background electrolyte (BGE). MeCN (20 %, *v*/*v*) was also added to the water plug to reduce conductivity, thus substantially enhancing sensitivity.

In-line back-extraction (ILBE) was performed with a water–organic drop hanging at the tip of the capillary [29]. In this case, the capillary was filled with acidic BGE (acceptor phase), and then 13 nL octanol was injected. After injection, the tip of the capillary was immersed in the urine sample, which had previously been made alkaline, and a backpressure was applied from the outlet to the inlet, forming a small drop of the acceptor phase that was covered with a thin organic layer hanging at the tip. After extraction, the acceptor phase was injected into CE. This configuration is well adapted to saline samples, for example urine; however, it is hardly achievable on a commercial CE instrument [26].

To enhance the transfer of analytes between the sample and organic phase, use of carriers with LLLME was envisaged by Choi et al. in 2011, in so-called carrier-mediated single-drop microextraction (CM-SDME) [30]. Amino acids were extracted from urine by use of nonane-1-sulfonic acid as carrier. Addition of this negatively charged carrier at a low pH with positively charged amino acids enabled the formation of a neutral ion pair that could be extracted into the organic phase. Octanol was chosen as the extracting phase because of its capacity to form hydrogen bonds with the ion pair. CM-SDME enabled 120-fold sensitivity improvement compared with CZE without SDME.

Drop stability improvement

Although the above-mentioned LLLME technique [28] has been shown to be fast, simple, inexpensive, and sensitive, it clearly suffers from drop instability. Therefore, in the same year, Fang et al. developed centrifuge microextraction (CME), which combines desalting, preconcentration, and removal of macromolecular contaminants and other interfering components in a single step [31]. After pH adjustment and addition of NaCl for the salting-out effect, 1 mL urine was mixed with 50 μL toluene and centrifuged at 10,000 rpm for 10 min. A lower-density water-immiscible solvent was chosen so the acceptor phase was at the top of the sample. During centrifugation, the centrifugal force applied by the rotor led to sedimentation of

Table 1 Liquid-based microextraction techniques used in combination with CE in bioanalysis

Analyte(s)	Matrix	ME	Sample volume	Organic solvent(s)	Organic solvent(s) volume	Analysis	PF/LOD	Ref.
Cocaine Thebaine	Urine	OLBE	8 mL	$CHCl_3$	4 μL	FASI–CZE–UV	120–340/2–10 $ngmL^{-1}$	[28]
Basic amines	Urine	ILBE	1.8 mL	*n*-octanol	13 nL	CZE–UV	1,000/0.5 $ngmL^{-1}$	[29]
Alkaloids	Urine	LLLME	4 mL	*n*-octanol	350 μL	MEKC–UV	1,583–3,556/0.2–1.5 $ngmL^{-1}$	[128]
Cyanide	Urine, saliva	HS-SDME	4.5 mL	–	–	CZE–UV after derivatization	58/0.08 μmol/L	[37]
Doping agents	Horse urine	HS-SDME	10 mL	$CHCl_3$–MeOH 90:10	5 μL	OT–CEC–UV	38–102/0.9–17.6 $ngmL^{-1}$	[38]
Toxic drugs	Urine	DLLME	4 mL	*i*-PrOH–CH_2Cl_2 70:30	2 mL 20 μL (*i*-PrOH, SL)	CZE–ESI–TOF MS	75–100/0.1–10 $ngmL^{-1}$	[40]
Serotonin Creatinine	Urine	USAEME	5 mL	Ethyl acetate	500 μL	CZE–UV	360/7.9 $nmolL^{-1}$ and 13.3 $\mu molL^{-1}$	[43]
Ephedrine derivatives	Urine, serum	CME	1 mL (urine) 20 μL (serum)	Toluene	50 μL	FASI–CZE–UV	3,800/0.15–0.25 $ngmL^{-1}$	[31]
Steroid hormones	Urine	CME	1.3 mL	Cyclohexane	100 μL	MEKC–UV	500/5–15 $ngmL^{-1}$	[32]
Alkaloids	Urine	DSDME	3.5 mL	*n*-octanol	< 60 μL	CZE–UV	231–524/8.1–14.1 $ngmL^{-1}$	[34]
Amino acids	Urine	CM-SDME	20 mL	*n*-octanol	24 nL	CZE–UV	120/70–500 $nmolL^{-1}$	[30]
NSAIDs	Urine	μ-SLM	4 mL	Dihexyl ether and MeOH	n.d.	MEKC–UV	1.2–1.7 μgmL^{-1}	[129]
Nitroimidazoles	Pig liver tissues	μ-SLM	0.5-5 g	2-Phenylpropane	n.d.	MEKC–UV	0.01–0.99 μgmL^{-1}	[47]
Bambuterol	Plasma	μ-SLM	500 μL	MeOH	n.d.	CZE–UV	n.d.	[130]
Bambuterol	Plasma	μ-SLM	350 μL	MeOH	n.d.	CZE–UV	14	[46]
Amino acids	Serum, plasma	μ-SLM	12.5 μL	ENB–DEHP	2.5 μL	CZE–C^4D	0.75–2.5 $\mu molL^{-1}$	[48]
Methamphetamine	Urine, serum	HF-LLLME	2.5 mL	1-octanol	n.d.	CZE–UV	75/5 $ngmL^{-1}$	[49]
Organomercury	Hair	HF-LLLME	12 mL	Bromobenzene	n.d.	LVSS–CZE–UV	2,610–4,580/0.03–0.14 μgmL^{-1}	[57]
NSAIDs	Urine	HF-LLLME	2.5 mL	Dihexyl ether	25 μL	CZE–UV	75–100/1 $ngmL^{-1}$	[52]
Methamphetamine Naproxen	Urine, plasma	HF-LLLME	4.0 mL (urine) 2.5 mL (plasma)	*n*-octanol	n.d.	CZE–UV	30–125	[50]
Citalopram Desmethylcitalopram	Plasma	HF-LLLME	1 mL	Hexyl ether	n.d.	CZE–UV	25–30/5 and 5.5 $ngmL^{-1}$	[54]
Mianserin	Plasma	HF-LLLME	0.5 mL	Di-*n*-hexyl ether	n.d.	CZE–UV	4 $ngmL^{-1}$	[131]
Citalopram Desmethylcitalopram	Plasma	HF-LLLME	1 mL	Dodecyl acetate	n.d.	CZE–UV	19–31/1.4–3.4 $ngmL^{-1}$	[53]
Methamphetamine Citalopram	Urine, plasma Whole blood	HF-LLLME	2.5 mL	Hexyl ether	n.d.	CZE–UV	95–145/2 $ngmL^{-1}$	[51]
Basic drugs	Plasma	HF-LLLME	250 μL	Dihexyl ether and MeOH	n.d.	CZE–UV	<20 $ngmL^{-1}$	[55]
Antidepressants	Human milk	HF-LLLME	500 μL	Polyphenylmethylsiloxane	n.d.	CZE–UV	14–23/<50 $ngmL^{-1}$	[56]
Rosiglitazone	Plasma, urine	HF-LLLME	5.0 mL (urine) 2.5 mL (plasma)	Dihexyl ether	n.d.	CZE–UV	280/2.83 $ngmL^{-1}$	[132]
Tricyclic antidepressants	Plasma	HF-LLLME	250 μL	Dihexyl ether	10 μL	CZE–UV	0.02–0.03 $\mu molL^{-1}$	[133]

Table 1 (continued)

Analyte(s)	Matrix	ME	Sample volume	Organic solvent(s)	Organic solvent(s) volume	Analysis	PF/LOD	Ref.
Cyanide	Urine, saliva	HF-HS-LPME	4.5 mL	–	–	CZE–UV	0.01 $\mu mol L^{-1}$	[134]
Polar drugs	Plasma, urine	CM-LPME	100 µL	1-octanol	n.d.	CZE–UV	10/< 1 $\mu g mL^{-1}$	[59]
Basic drugs	Plasma	CM-LPME	50 µL	*n*-octanol	n.d.	CZE–UV	<0.5 $\mu g mL^{-1}$	[60]
Basic drugs	Plasma, urine	EME	100 µL	NPOE	~15 µL	CZE–UV	n.d.	[62]
Basic drugs	Plasma, whole blood	EME	500 µL	1-ethyl-2-benzene	n.d.	CZE–UV	<0.5 $\mu g mL^{-1}$	[64]
Angiotensins	Plasma	EME	500 µL	1-octanol–DEHP	n.d.	CZE–UV	<2.5 $ng mL^{-1}$	[66]
Basic drugs	Plasma, urine Human milk	EME	1 mL	1-isopropyl-4-nitrobenzene	n.d.	CZE–UV	Up to 22	[65]
Amino acids	Serum, plasma Whole blood,	EME	36 µL	ENB–DEHP	n.d.	CZE–C^4D	0.15–10 $\mu mol L^{-1}$	[67]
Lithium	Urine Serum, plasma Whole blood	EME	35 µL	1-octanol	n.d.	CZE–C^4D	9 $nmol L^{-1}$	[68]
Basic drugs Amino acids	Urine, serum	EME	875 µL (urine) 145 µL (serum)	ENB/DEHP	n.d.	CZE–UV	1–4 $ng mL^{-1}$ (bases) 0.6–3 $\mu mol L^{-1}$ (acids)	[135]
Amlodipine	Plasma, urine	EME	1 mL (serum) 1.5 mL (urine)	NPOE	n.d.	CZE–UV	Up to 150/3 $ng mL^{-1}$	[69]
Trimipramine	Plasma, urine	EME	1.25 mL (serum) 2.5 mL (urine)	NPOE	n.d.	CZE–UV	120–149/8–10 $ng mL^{-1}$	[70]
Basic drugs	Urine	EME	10 µL	NPOE	1 µL	CZE–UV	< 1 $\mu g mL^{-1}$	[71]
Inorganic and organic mercury	Hair	PT-LLLME	0.25 g	Toluene MeCN Acetone	200 µL (Toluene) 225 µL (MeOH)	LVSS–CZE–UV	12,138	[58]
Basic and acidic drugs	Urine	DMD-LPME	15 µL	1-octanol	1 µL	MCE–LIF	2	[72]
Tramadol, paracetamol and metabolites	Urine	DMD-LPME	4 µL	1-octanol	~1 µL	MCE–ESI–MS	2/9.3 $nmol L^{-1}$ for model compound	[73]

n.d., not defined

LOD is determined at a signal-to-noise ratio of 3

macromolecules whereas diffusion enabled transfer of the targeted compounds to the acceptor phase. The supernatant was directly injected in CE–UV with FASI, leading to a limit of detection (LOD) of 0.15 $ng mL^{-1}$. CME has also been used for analysis of steroids (e.g., testosterone and progesterone) in urine by micellar electrokinetic chromatography (MEKC) [32].

In 2006, Yangcheng et al. proposed directly suspended droplet microextraction (DSDME) to further improve drop stability. Here, the microdroplet of solvent is suspended at the top in the center of the aqueous sample before sampling [33]. A symmetrical rotated flow field is created by a stirring bar that is placed on the bottom of the cylindrical sample cell to ensure the droplet suspension. This rotation also intensifies transfer of analytes to the inside of the droplet. DSDME has been combined with single-drop back-extraction and CE for analysis of alkaloids in urine samples [34]. After the first extraction in a large microdrop (approx. 60 μL) of *n*-octanol, alkaloids were back-extracted in a 1-μL aqueous drop that was immersed in the organic phase droplet. PFs of greater than 500 were achieved with lower solvent consumption and shorter extraction time than those of LLLME.

Introduced by Theis et al. [35] in 2001, headspace single-drop microextraction (HS-SDME) has excellent extraction and preconcentration performance for volatile compounds. With a suspended drop in the gaseous phase (headspace), this method enables rapid stirring of an aqueous sample, for a shorter analysis time, without affecting drop stability. Moreover, non-volatile matrix interferences are reduced or eliminated [19, 26, 36]. HS-SDME has also been used in combination with CE analysis with in-drop derivatization. Free cyanide was solventlessly extracted from smoker and non-smoker urine and saliva [37], using water to extract volatile and water-soluble compounds. An aqueous 5-μL drop containing Ni(II)–NH_3 as derivatization agent for CE analysis was used for the extraction. In the basic acceptor phase, cyanide reacted with Ni^{2+} to form a stable $Ni(CN_4)^{2-}$ complex analyzed by CE–UV at 257 nm. Water-based HS-SDME was very selective, despite the rather universal detection wavelength, because the non-volatile interferents remained unaffected in the sample. HS-SDME with a chloroform–MeOH mixture as extracting drop has also been used to extract seven toxic compounds from horse urine samples at room temperature, before analysis by open tubular capillary electrochromatography (OT-CEC) [38].

Dispersive liquid–liquid microextraction (DLLME)

In DLLME, which was first introduced by Rezaee et al. in 2006, the extracting solvent is mixed with a dispersing solvent that is miscible both with the former and with the aqueous sample [39]. The mixture is rapidly injected into the sample with a syringe, producing high turbulence that leads to the formation of tiny droplets. Because of the large surface area between the extracting droplets and sample, the extraction time is drastically reduced. After centrifugation, the sedimented phase at the bottom of the tube is collected and either injected directly or evaporated to dryness before reconstitution and injection.

DLLME combined with CE and time-of-flight mass spectrometry (TOF/MS) was used for qualitative toxicological screening of urine samples [40]. An experimental design strategy was used to increase the extraction efficiency. CH_2Cl_2 and *i*-PrOH were selected as extracting and dispersing solvents, respectively, with a total volume of 2 mL. Because of a high PF (more than 130) and the high sensitivity and selectivity of CE–TOF/MS, LODs down to the *sub*-$ng mL^{-1}$ range were obtained for more than 30 toxic basic compounds and their main metabolites and confirmed by real case analysis.

Extraction improvement

One of the main disadvantages of DLLME is the need to use a dispersing solvent to create an emulsion, which can reduce the partition coefficient of the analytes in the extracting phase and increase total solvent consumption. The dispersing solvent can be substituted by using ultrasound to achieve ultrasound-assisted emulsification-microextraction (USAEME) [41]. Based on previous work on ultrasound-assisted sample preparation [42], USAEME is beneficial for promoting emulsion formation, extending the contact surface between both phases, and reversing the potential coalescence effect. Increasing the temperature also enables efficient and fast extraction [41]. A serial USAEME procedure was developed for analysis of creatinine and serotonin in urine samples [43]. Five hundred microliters of ethyl acetate was added to 5 mL urine and the sample was immersed in an ultrasonic bath for 5 min at 40 Hz. The emulsion was centrifuged, and the organic supernatant mixed with 25 μL 0.1 $mol L^{-1}$ HCl. Back-extraction was performed by 3-min ultrasonication at 40 Hz. After centrifugation, the sedimented acceptor phase was collected and injected by use of a pH-mediated stacking procedure. With serial USAEME and sample stacking, a PF of 360 was obtained for serotonin.

Hollow-fiber-based liquid-phase microextraction (HF-LPME)

The chemical principle of HF-LPME is derived from supported-liquid membrane (SLM) extraction, which was previously developed by Jönsson and coworkers [44]. In SLM, analytes are extracted through a flat porous polymeric membrane sheet with continuous sample pumping. SLM was first miniaturized ("μ-SLM") in 1996 by Jönsson and coworkers [45] and applied to the analysis of bambuterol in plasma samples that were continuously pumped to on-line

SLM–CZE [46]. An in-line SLM approach with a Teflon micromembrane unit glued to a plastic microtube integrated in the CE vial was developed by Nozal et al. for analysis of nitroimidazoles in pig liver tissues homogenized in water [47]. Very recently, Kuban and Bocek proposed on-line μ-SLM-CE with a planar SLM screwed between two PTFE blocks to determine amino acids in plasma or serum [48]. This home-built set-up did not require additional pumps.

In contrast to μ-SLM, HF-LPME is performed without any pumping device. It was introduced in 1999 by Pedersen-Bjergaard and Rasmussen [49]. In HF-LPME, the extracting phase is placed inside the lumen of a porous polypropylene fiber (pore size 0.2 μm) of minimal dimensions used in different configurations, e.g., U-shaped, rod-like, 96-well, or directly connected to a microsyringe [21, 44]. The polymeric fiber, which is compatible with a broad range of organic solvents, enables use of a larger extraction volume compared with SDME and acts as a physical barrier between phases, avoiding undesirable emulsions and enhancing cleanup efficiency [19]. HF-LPME can be performed in either two or three-phase systems. In three-phase systems, referred to as hollow-fiber-based liquid–liquid–liquid microextraction (HF-LLLME), supported liquid membrane microextraction (SLMME), or, rather improperly, LPME, the analytes are extracted from the aqueous sample through the organic film (a few microliters) that is present in the pores of the aqueous acceptor phase in the lumen of the hollow fiber. HF-LLLME is well suited to extraction of polar or ionizable compounds and particularly suitable for CE analysis.

Hollow-fiber-based liquid–liquid–liquid microextraction (HF-LLLME)

Pedersen-Bjergaard and Rasmussen with co-workers have developed many applications of HF-LLLME in combination with CE. Methamphetamine [49–51], non-steroidal anti-inflammatory drugs (NSAIDs) [52], naproxen [50], citalopram and metabolites [51, 53, 54], and a variety of basic drugs [55] have been successfully extracted from urine and serum or plasma. HF-LLLME has also been used to extract antidepressants from human milk [56]. Human milk is characterized by high protein, fat, and carbohydrate content, which can affect the recovery and repeatability of the extraction procedure. Because of interaction of antidepressants with fat and proteins, recovery from milk was lower than from water. Thus, PP was implemented, with addition of hydrochloric acid to the sample before centrifugation and extraction to remove the fat-rich layer and release unbound drugs, leading to recovery of 50–70 %. Li et al. used HF-LLLME for extraction of organomercury from human hair, with the fiber pores impregnated with bromobenzene [57]. Hair samples were first rinsed with detergent and acetone, and air-dried before cutting and leaching. The leached solution was centrifuged, and the supernatant collected for HF-LLLME. An aqueous acceptor phase containing L-cysteine for organomercury complexation was injected with large volume sample stacking (LVSS), enabling enrichment of more than 4,000.

Extraction improvement

A new LPME-based technique referred to as phase-transfer-based liquid–liquid–liquid microextraction (PT-LLLME) was developed in 2011 by Li et al. for extraction of organic and inorganic mercury from hair [58]. In this homemade set-up, a porous, hydrophilic, nylon-membrane-supported extraction tip was built and used with 15 μL aqueous acceptor phase. MeCN and dodecylamine were added to the sample before extraction as intermediate solvent and complexing reagent, respectively. MeCN improved the dispersion of water-immiscible dodecylamine in the aqueous sample to ensure maximum contact with the mercury. Compared with mercury extraction by HF-LLLME, PT-LLLME provided the potential for simultaneous speciation of inorganic and organic mercury and improved the sensitivity with enhanced extraction efficiency.

Use of carriers, also used in SDME (section “Liquid–liquid–liquid microextraction (LLLME)”, subsection “Extraction improvement”), was first introduced in 2003 by Ho et al. in the so-called carrier-mediated liquid-phase microextraction (CM-LPME) to enhance extraction recovery of polar or ionic analytes [59]. The carriers form lipophilic complexes with the target analytes, promoting the transport of the analytes through the organic membrane. Polar basic compounds could be extracted from plasma and urine samples, through the 1-octanol layer into the aqueous acceptor phase, with good recovery, after addition of sodium octanoate (ion-pair reagent) to the sample. The pH of the sample had to be adjusted so the analytes and carrier were ionized in such a way to enable the formation of ion-pair complexes that could diffuse through the membrane. Numerous carriers, including organic borates, phosphates, sulfates, and carboxylic acids, were investigated at different concentrations with a special emphasis on their compatibility with plasma samples [60]. Bromothymol blue (sulfate carrier) resulted in the best recovery from the plasma samples. Interestingly, recovery was enhanced when sodium sulfate was added to the sample to reduce matrix effects.

In 2005, Lee and coworkers developed hollow-fiber-protected headspace liquid-phase microextraction (HF-HS-LPME), in which the hollow fiber protected and held the extractant droplet in the headspace [61]. The surface area between the organic and acceptor phases was dramatically enhanced compared with HS-SDME (section “Liquid–liquid–liquid microextraction (LLLME)”, subsection “Drop

stability improvement"), increasing the extraction efficiency. HS-HF-LPME was used to extract free cyanide from urine and saliva with a simultaneous in-fiber derivatization to form a stable $Ni(CN)_4^{2-}$ complex. Lower LODs (0.01 μmolL^{-1} versus 0.08 μmolL^{-1}) and similar recovery (90–105 %) were obtained compared with HS-SDME [37]. HF-HS-LPME is an effective alternative to HS-SDME for quantitative analysis of volatile compounds.

Throughput improvement

In 2006, Pedersen-Bjergaard and Rasmussen proposed use of an electrically-driven force to aid extraction of charged compounds and to speed HF-LLLME [62, 63]. This technique was first referred to as "electro membrane isolation" (EMI) and was later termed electro membrane extraction (EME). Two platinum electrodes are placed in the sample solution and in the aqueous acceptor phase in the lumen of the fiber. A potential (typically 300 V) is applied, and charged analytes migrate through the membrane toward the oppositely charged electrode in the acceptor solution in less than 5 min. Interesting clean-up, enrichment, and isolation of basic compounds with 2-nitrophenyl octyl ether (NPOE) as organic solvent were observed with high extraction recovery (>70 %) from plasma and urine.

Pedersen-Bjergaard, Rasmussen, and co-workers showed the benefits of EME for analysis of basic drugs (e.g., analgesics, antidepressants, and antiepileptics) in plasma and whole blood [64], or urine and human milk [65]. They also evaluated the potential of EME as a fast and effective extraction technique for peptides (angiotensin as the model peptide) in plasma [66].

Other groups evaluated EME for a variety of applications, for example extraction of amino acids [67], lithium [68], amlodipine enantiomers [69], and trimipramine enantiomers [70] from urine, plasma or serum, or whole blood.

A miniaturized form of EME, termed drop-to-drop LPME, has been proposed for extraction of basic drugs from urine and plasma [71]. A small well with a volume of 15 μL was pressed into 5-cm^2 aluminium foil connected to the power supply's positive outlet. The well, containing 10 μL sample, was covered with the membrane and a 10-μL acceptor droplet. Recovery of 33–47 % was obtained with excellent clean-up, short extraction time, and very low solvent and sample consumption.

In 2010, this miniaturization was built upon with the development of on-line droplet–membrane–droplet LPME (DMD-LPME) [72]. The extraction set-up was the same as in Ref. [71] and was combined on-line with microchip capillary electrophoresis (MCE) with fluorescence detection. DMD-LPME was directly compatible with MCE because of the very low acceptor phase volume. After 5 min, analysis of two model analytes spiked in blank urine led to recovery of 15 and 25 %, which was lower than from aqueous standards. However, DMD-LPME was found to be competitive for high-throughput analysis, because of the high extraction speed and its feasibility for coupling with rapid microfluidic analysis. DMD-LPME has also been combined with MCE for drug metabolism studies with ESI-triple quadrupole MS detection [73]. Compared with SPE, DMD-LPME enabled faster analysis and higher selectivity for phase I metabolites.

Solid-based microextraction techniques

SPE is the most widely used technique for clean-up, preconcentration, and selective extraction. Over the last few decades, a large variety of commercial silica-based or polymeric sorbents (e.g., normal-phase, reversed-phase, ion-exchange mode, mixed-mode, and, more recently, molecularly imprinted polymers (MIP), monoliths, and restricted-access media, RAM) have been developed to enable extraction of a variety of analytes with divergent chemical structure and polarity, with careful attention to higher loading capacity and efficiency. SPE can be automated easily, furnishes high recovery, and is claimed to be highly selective in relation to matrix interferences [2, 17]. However, conventional SPE has some limitations, for example relatively high solvent consumption and batch-to-batch variability [16, 17]. A significant amount of progress has been made with SPE to substantially reduce solvent consumption and increase sample throughput, for example the advent of column-switching systems with on-line extraction, or the multi-well plate format. Another substantial step was achieved with SPE miniaturization and the development of new microextraction techniques (solid-based MEs), for example solid-phase microextraction (SPME), microextraction by packed sorbent (MEPS), and stir-bar-sorptive extraction (SBSE), all of which have several advantages and result in significantly improved sample preparation. The solid-based MEs used in combination with CE are presented in Fig. 2, and all the bioanalytical applications are listed in Table 2.

Solid-phase microextraction (SPME)

SPME was introduced in 1990 by Arthur and Pawliszyn [74]. A small amount of sorptive, homogenous, non-porous extracting phase dispersed on the surface of or inside a solid support is exposed to the sample for a specific period of time until equilibrium is reached [75, 76]. The main commercially used sorbents are polydimethylsiloxane (PDMS) for rather non-polar or volatile compounds and polyacrylate (PA), PDMS–divinylbenzene (PDMS–DVB), or Carbowax–divinylbenzene (CW–DVB) for polar compounds. Extraction can be performed in two main formats: fiber SPME and in-tube SPME.

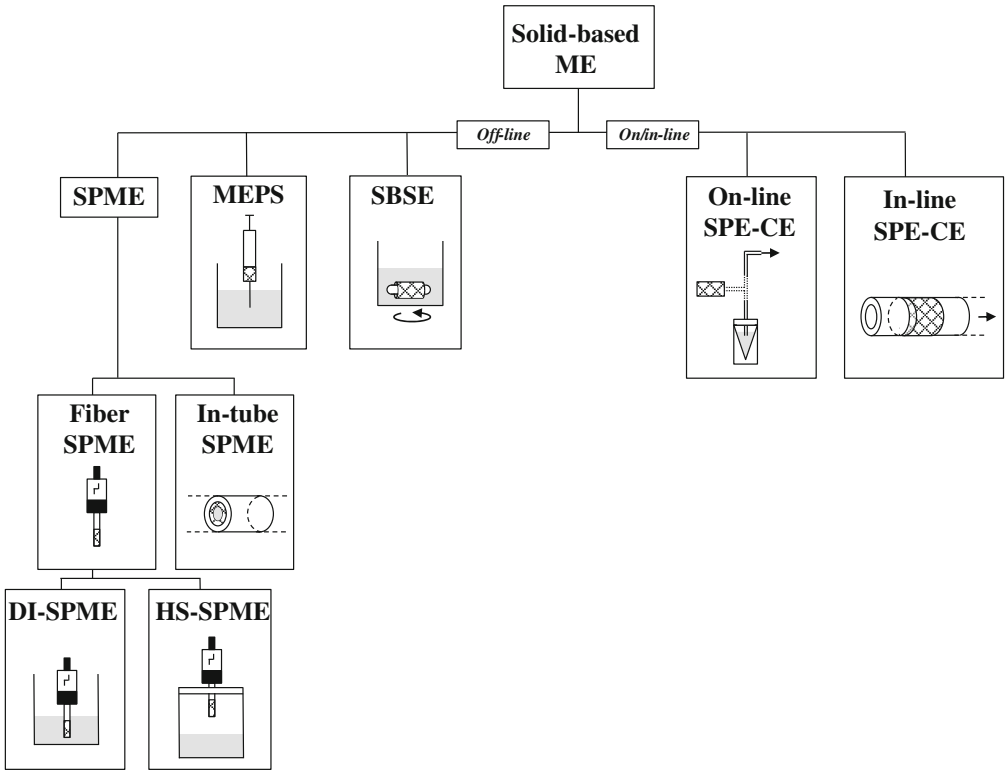

Fig. 2 Classification of solid-based microextractions used in combination with CE. *Light gray*, aqueous phase; *cross hatched*, solid support

Fiber solid-phase microextraction (fiber SPME)

In fiber SPME, the sorbent (variable film thickness) is coated on the external surface of a fused-silica fiber tip as an appropriate polymeric stationary phase. The device, a modified syringe, consists of a fiber assembly with the built-in fiber inside the needle and an assembly holder. A plunger is used to move the coated fiber inside or outside the needle [19, 77]. Two extraction modes can be used with fiber SPME: direct immersion of the fiber in the aqueous sample (DI-SPME) or headspace extraction (HS-SPME), which was first described in 1993 [78].

Direct-immersion fiber solid-phase microextraction (DI-SPME)

DI-SPME entails direct immersion of the fiber into the aqueous sample with consequent stirring, enabling transfer of non-volatile analytes into the coating [76]. Barbiturates and benzodiazepines have been extracted by use of a PA-coated fiber that was immersed in 10 mL urine for 2 h at 60 °C. After extraction, the targeted drugs were desorbed into 20 μL MeCN for 30 min and analyzed by MEKC on neutral polyacrylamide-coated capillaries [79, 80].

Headspace fiber solid-phase microextraction (HS-SPME)

HS-SPME has been shown to be advantageous, mainly for volatile compounds, because of its higher speed, higher recovery, greater selectivity, longer fiber lifetime, and lower fiber contamination than for DI-SPME, but it is only suitable for highly volatile compounds [76, 81]. Instead of using a conventional PDMS, PA, or poly(vinyl chloride) fiber, Zeng and coworkers developed HS-SPME with a calix{4}arene fiber [82], for propranolol determination, and co-poly (butyl methacrylate–hydroxy-terminated silicone oil), using a sol–gel coating, for extraction of ephedrine derivatives in urine [83]. After a second back-extraction step in a MeCN–water solution (less than 20 μL MeCN), the analytes were injected with the FASI stacking method, leading to important PFs.

In-tube SPME

In-tube SPME, which was introduced in 1997, was primarily developed to overcome the inherent problems of fiber SPME, i.e., fiber fragility, low sorption capacity, and bleeding of fiber coatings, and to provide an automation option [84]. In this method, targeted compounds are directly extracted into the internally coated stationary phase of a fused-silica capillary, enabling on-line coupling with CE [85]. In-tube SPME is a type of so-called capillary MEs, which also include open-tubular trapping, wire-in-tube SPME, fiber in-tube SPME, sorbent-packed capillary in-tube SPME, and monolithic capillary in-tube SPME [75, 84, 86]. Capillary MEs are distinguished from the composition of the extraction stationary phase (fiber, polymer, sorbent) and its packing [86] and can be used on-line with CE.

Table 2 Solid-based microextraction techniques used in combination with CE in bioanalysis

Analyte(s)	Matrix	ME	Sample volume	Organic solvent(s)	Organic solvent(s) volume	Analysis	PF/LOD	Ref.
Ephedrine Pseudoephedrine	Urine, serum	MIP-SPME	5 mL	Toluene Ethanol MeOH	5 mL 7 mL n.d.	CZE–UV	0.96 and 1.1 $ngmL^{-1}$ (water sample)	[136]
Ephedrine derivatives	Urine	HS-SPME	5 mL	MeCN	16 µL	FASI–CZE–UV	3–5 $ngmL^{-1}$	[83]
Propranolol enantiomers	Urine	HS-SPME	5 mL	MeCN	10 µL	FASI–CZE–UV	8–10 $ngmL^{-1}$	[82]
Barbiturates	Human urine, bovine serum	DI-SPME	3.5 mL	–	–	CZE–UV	Up to 60/0.1–0.3 μgmL^{-1} (urine) and 1 μgmL^{-1} (serum)	[137]
Barbiturates, benzodiazepines	Urine	DI-SPME	10 mL	MeCN	20 µL	MEKC–UV	<1 μgmL^{-1}	[79, 80]
Amphetamines	Urine	Monolithic in-tube SPME	40 µL	MeOH	n.d.	EK–CZE–UV	25–34 μgmL^{-1}	[88]
Opiates	Urine	Monolithic in-tube SPME	1 mL	MeOH	325 µL	EK–CZE–UV	6.6–19.5 $ngmL^{-1}$	[89]
Ephedrine Pseudoephedrine	Urine, plasma	Monolithic in-tube SPME	1 mL	MeOH MeCN	300 µL (MeOH) ~100 µL (MeCN)	LVSS–CZE–UV	5.3–8.4 $ngmL^{-1}$	[90]
Angiotensin II receptor antagonists	Urine	Monolithic in-tube SPME	2 mL	MeCN	500 µL+BGE	CZE–UV	15–20 $ngmL^{-1}$	[91]
Propranolol enantiomers	Urine	In-tube SPME	1 mL	MeOH MeCN	35 µL (MeOH) 100 µL (MeCN)	CEC–UV	4 and 7 $ngmL^{-1}$	[138]
Tricyclic antidepressants	Urine	Fiber-in-tube SPME	1 mL	MeCN	1.8-2.2 µL+BGE	CZE–UV	>100/44–153 $ngmL^{-1}$	[87]
Caffeine, paracetamol, acetylsalicylic acid	Bovine plasma	RAM capillary in-tube SPME	<10 µL	MeOH	n.d.	CZE–UV	0.3–1.9 $ngmL^{-1}$	[92]
PAHs	Fish bile	SBSE	0.3 g	MeCN	150 µL	MEKC–UV	2–11 μgmL^{-1}	[99]
Fluoroquinolones	Urine	MEPS	48 µL	MeCN MeOH	n.d.	NACE–ESI–MS	6.3–10.6 μgmL^{-1}	[96]
Anesthetic drugs	Plasma	MEPS	200 µL	MeCN MeOH	n.d.	NACE–ESI–MS	10.4–15.2 μgL^{-1} (free) 0.6–1.6 $ngmL^{-1}$ (total)	[95]
Opioids	Urine	In-line SPE	~60 µL	MeOH *i*-PrOH	~30 nL (MeOH) ~45 µL (*i*-PrOH)	CZE–ESI–MS	0.013–0.210 $ngmL^{-1}$	[139]
Methionine encephalin	Cerebrospinal fluid	In-line SPE	3.2 µL	MeCN MeOH	40 nL+conditioning (MeCN) ~90 µL (MeOH, SL)	CZE–ESI–MS	40/1 $ngmL^{-1}$	[117]
Enkephalin peptides	Cerebrospinal fluid	On-line SPE	100 µL	MeCN	1.7 µL (elution and rinse) ~20 µL (SL)	CZE–ESI–IT/MS	1,000/1.5–3 $ngmL^{-1}$	[103]
Cephalosporins	Cow plasma	On-line SPE	50 µL	MeCN	1.8 µL	CZE–UV	100 $ngmL^{-1}$	[104]
Opioid peptides	Plasma	In-line SPE	200 µL	MeOH *i*-PrOH MeCN	~40 nL (MeOH, elution) ~30 µL (*i*-PrOH, SL) 1200 µL (MeCN, PP)	CZE–ESI–IT/MS	100–10,000/0.1–10 $ngmL^{-1}$	[112]
Neuropeptides	Plasma	In-line SPE	200 µL	MeOH *i*-PrOH MeCN	~40 nL (MeOH, elution) ~50 µL (*i*-PrOH, SL) 1400 µL (MeCN, PP)	CZE–ESI–IT/MS	100–10,000/0.1–10 $ngmL^{-1}$	[111]
Opioid peptides	Plasma	In-line SPE	200 µL	MeOH *i*-PrOH	~40 nL (MeOH, elution) ~10 µL (*i*-PrOH, SL)	tITP–CZE–TOF/MS	Up to 5,000/0.1 $ngmL^{-1}$	[113]

Table 2 (continued)

Analyte(s)	Matrix	ME	Sample volume	Organic solvent(s)	Organic solvent(s) volume	Analysis	PF/LOD	Ref.
				MeCN	1400 µL (MeCN, PP)			
Opioid peptides	Plasma	In-line SPE	200 µL	MeOH *i*-PrOH	<100 nL (MeOH, elution) <70 µL (*i*-PrOH, SL)	CZE–ESI–IT/MS	10–100/0.1–1 ngmL^{-1}	[110]
				MeCN	1400 µL (MeCN, PP)			
Endormorphins	Plasma	In-line SPE	200 µL	*i*-PrOH MeCN	~20 µL (*i*-PrOH, SL) 1400 µL (MeCN, PP)	CZE–ESI–TOF/MS	100/1 ngmL^{-1}	[114]
Neurotransmitters	Urine	In-line SPE	<1 µL	-	-	CZE–UV	Up to 462/3.7–4.3 ngmL^{-1}	[118]
Caffeine	Urine	In-line SPE	<1 µL	-	-	CZE–UV	1,500–1,900/0.5–0.7 ngmL^{-1}	[119]
Escitalopram	Urine	In-line SPE	<2.25 µL	MeCN MeOH	n.d. (MeCN, BGE and elution) ~25 µL (MeOH, SL)	CZE–ESI–TOF/MS	10 ngmL^{-1}	[140]
Sulfonamides	Urine, serum	On-line SPE	1 mL	THF MeCN	600 µL (THF, elution) 200 µL (MeCN, PP)	CZE–UV	0.05–0.1 µgmL^{-1} (urine) 0.05–0.3 µgmL^{-1} (serum)	[100]
NSAIDs	Urine, serum	On-line SPE	1 mL	MeCN	<1 mL (PP and elution)	CZE–UV	0.05–0.1 µgmL^{-1} (urine) 0.1–1 µgmL^{-1} (serum)	[101]
Tricyclic antidepressants	Urine, serum	On-line SPE	0.5 mL	MeOH MeCN	<1 mL (elution, wash, BGE composition)	NACE–UV	40–80 ngmL^{-1} (urine) 60–100 ngmL^{-1} (serum)	[102]
Endogenous biomarkers	Urine	On-line SPE	2.5 mL	MeCN MeOH	<1 mL (elution, wash, BGE composition)	CZE–UV	0.14–4.50 µgmL^{-1}	[141]
3-Nitrotyrosine	Rat urine	In-line SPE	<200 µL	MeCN MeOH	n.d.	CZE–UV	100/4.4 µmolL^{-1}	[142]
Triazine herbicides	Urine	In-line SPE	1 mL	MeCN MeOH	~30 nL (MeCN, elution) ~15 µL (MeOH, rinse)	CZE–UV	0.2–0.6 µgmL^{-1}	[115]

n.d., not defined

LOD is determined at a signal-to-noise ratio of 3

Fiber in-tube SPME has been used for analysis of four tricyclic antidepressant drugs (TCAs) in urine [87]. A 10-mm-long Zylon fiber filling a capillary placed inside a 0.25-mm i.d. Teflon tube was connected on-line to the CE system. After continuous pumping of the sample, TCAs were desorbed with a few microliters of MeCN, directly transferred to a cross connector, and separated by CE, leading to 100-fold greater sensitivity.

Feng and co-workers used monolith capillary in-tube SPME with poly(methacrylic acid–ethylene glycol dimethacrylate) for extraction of amphetamines [88], opiates [89], ephedrine and pseudoephedrine [90], and angiotensin II receptor antagonists [91] from urine and plasma samples. Some of these applications were performed with an adapted device composed of a regular plastic syringe and a monolithic capillary connected by a pinhead (polymer monolith microextraction, PMME) [89, 91]. In monolith capillary in-tube SPME, a single piece of monolith with a double-pore structure enables use of high flow rates with a low generated pressure through the capillary, leading to high throughput [84, 86]. An alternative approach is the use of continuous bed RAM in-tube SPME, which enabled simultaneous protein separation from the matrix while directly extracting target analytes [92].

Microextraction by packed sorbent (MEPS)

MEPS was developed in 1993 and consists of a 100 to 250-μL syringe containing 1 to 4 mg of packed sorbent (inserted into the barrel of the syringe as a plug or between the barrel and needle as a cartridge). The sorbents are miniaturized to work with microliter bed volumes, enabling use of sample and elution volumes as low as 10 μL. All the commercially available SPE sorbents, including RAM and MIP, can be used in MEPS [17, 77, 93, 94].

Recently, Morales-Cid et al. used at-line and on-line coupled MEPS with CE–MS for determination of anesthetic drugs in plasma [95] and fluoroquinolones in urine [96]. In the first study, MEPS was performed with a 200-μL syringe containing 4 mg C_{18} packing. A microdialysis probe was connected to the needle of the MEPS syringe and the method was fully automated. Using 200 μL plasma and non-aqueous CE (NACE) analysis coupled with MS, LODs as low as 10 $ng mL^{-1}$ were reported for the free anesthetic drugs [95].

In the second study, the extraction step was directly integrated into a commercial CE system. The barrel insert and needle containing 4 mg C_{18} packing were fitted to the outlet position of the CE–MS cartridge and connected to a Teflon tube inside the cartridge working as a reservoir (300 μL) for conditioning, preconcentration, and elution. Using CE equipment pressures, samples were preconcentrated and extracted on-line before separation. Only 48 μL urine and 140 μL MeOH were required for conditioning and elution. The eluates were analyzed by NACE-MS to increase resolution and sensitivity. With this configuration, absolute recovery from urine ranged from 70 to 109 % with LODs of less than 10 $ng mL^{-1}$ [96].

Stir-bar-sorptive extraction (SBSE)

Based on the same extraction principle as SPME, SBSE was first developed in 1999 by Baltussen et al. to overcome the limited amount of extraction sorbent used in SPME [97, 98]. In SBSE, the extraction sorptive phase is coated (0.5 to 1-mm layer) on to magnetic stir bars (1 to 4 cm in length) composed of a magnetic rod surrounded by a glass jacket. During stirring of the aqueous sample (typically 30 to 240 min), analytes are extracted in accordance with their partition coefficients. Desorption can be performed thermally or by liquid desorption by organic solvent back-extraction.

There have been few applications of SBSE in combination with CE. Do Rosario et al. developed an SBSE–MEKC method for determination of polynuclear aromatic hydrocarbons (PAHs) in fish bile [99], but no applications in human bioanalysis have been found. Nonetheless, SBSE could be used for extraction of urine samples, because of the relatively large volumes of urine available and the long detection times required to achieve very low LODs of metabolites. However, commercial coated stir bars (Twisters; Gerstel, Mühlheim, Germany) are still limited to PDMS and PDMS–ethylene glycol phases, which are better suited to extraction of non-polar compounds.

SPE-CE

Off-line SPE is largely used in combination with CE, because of its ease of implementation. Over the past two decades, new set-ups have been developed to automate this process, increase sample throughput, and reduce solvent consumption. At-line coupling of SPE with CE is performed with a robotic arm interface or a modification of the replenishment system. Despite increased throughput, the same solvent quantities are used for sample preparation. More advantageous techniques are on-line and in-line SPE-CE, in which the liquid stream is shared between SPE and CE and analysis can therefore be achieved with relatively small volumes of organic solvents.

On-line SPE-CE

In on-line SPE-CE, an interface (vial, valve, or T-piece type) is used to directly connect the stream from the SPE part and the CE capillary. Because the SPE process is performed independently of CE analysis, no adsorption of the matrix components on to the capillary wall is observed, nor any perturbation of the electrophoretic process. However, peak broadening can be

observed because the desorption volume generally larger than the CE injection volume.

Veraart et al. developed on-line dialysis SPE–CE for analysis of sulfonamides [100] and NSAIDs [101] in urine and serum. The system comprised a dialysis unit, four switching valves, four high-pressure pumps, and a polymer-based SPE column. When dialysis SPE had been performed, a signal was sent to the CE system to transfer the analytes that could be analyzed. A THF–water mixture could be used for analyte desorption to avoid bubble formation [100] or a MeCN–water mixture could be used to ensure a good stacking effect during injection [101]. For serum analysis, a PP with MeCN and decanoic acid was first performed to disrupt drug–protein bonding. LODs in the ng mL^{-1} range were reported for urine samples analyzed by CE–UV. This dialysis set-up, with NACE, was also used for analysis of tricyclic antidepressants in urine and serum [102].

More recently, de Jong and co-workers proposed use of on-line SPE-CE with ion-trap (IT) MS detection for analysis of peptides in cerebrospinal fluid [103]. Enkephalin peptides were extracted on C_{18} sorbent from diluted cerebrospinal fluid and introduced into the CE system via a valve interface. Less than 2 μL MeCN was necessary for analyte desorption. The sensitivity was 1,000-fold better than that obtained by conventional CE–MS. This technique was applied to a tryptic digest of cytochrome *c*, and LODs were as low as 20 nmol L^{-1}, indicating the potential for proteomics. An alternative on-line SPE–CE–UV procedure was proposed for analysis of antibiotics (cephalosporins) in cow plasma with a T-split interface [104]. Part of the SPE eluate was injected and the rest of the sample was flushed to waste (split ratio 1:40). Before SPE–CE, PP with 10 % perchloric acid was performed for plasma samples, avoiding the use of organic solvent, which would reduce the breakthrough volume on the C_{18} SPE column or increase the total analysis time, because of evaporation and reconstitution. With these conditions, LODs were in the 50–100-ng mL^{-1} range, similar to those reported for other LC–UV methods.

In-line SPE-CE

In in-line SPE-CE, the SPE material is part of the CE capillary, and the potential is applied on the entire system during separation using either an open tubular capillary coated with SPE sorbent, a packed-bed sorbent retained with frits, silica- or polymer-based monoliths, or an impregnated membrane. Recently, carbon nanotubes, magnetic particles, or antibodies for immunoaffinity recognition have been successfully investigated. The overall SPE eluate is analyzed by CE, resulting in good recovery. Nevertheless, the latter greatly depends on the nature and volume of the elution solvent. Furthermore, because of direct transfer of the extraction eluate, adsorption of matrix components on to the capillary wall can affect the separation or clog the capillary [105–109].

Sanz-Nebot, Barbosa, and co-workers developed several applications of CE–ESI–MS with in-line SPE microcartridges [110–114]. In their homemade set-up, a CE capillary is cut into two pieces to enable insertion of an SPE microcartridge. Its body is coupled to both parts with a 0.5-cm polyethylene sleeve and equipped with 0.1-cm polyethylene frits after sorbent filling. The tight junction obtained means no adhesive sealing is necessary, and the modified capillary is fitted into commercial CE cartridges. This approach has been successfully applied to the analysis of opioid peptides [110, 112, 113] and neuropeptides [111] in plasma samples with C_{18} or other sorbents [110]. In a recent study, use of an immunoaffinity sorbent for the analysis of endormorphins in plasma by in-line SPE–CE–ESI-MS was also evaluated [114]. In this case, the previously developed microcartridge contained the immunoaffinity sorbent consisting of anti-endorphin antibodies that were covalently attached to activated hydrazide silica particles via carbohydrate groups. Immunoaffinity sorbents resulted in improved selectivity and extraction efficiency with a larger introduced sample volume. LODs as low as 1 ng mL^{-1} in standard solutions were achieved with a 100-fold PF compared with CE–MS, and LODs as low as 100 ng mL^{-1} were achieved for plasma after PP and filtration. However, some cross-reactivity against dynorphin, because of non-specific binding, was also observed.

MIPs, also, are regarded as highly selective synthetic materials with recognition sites that can specifically bind target analytes. Molecularly imprinted solid-phase extraction (MISPE) has been evaluated as an in-line SPE-CE technique for monitoring of triazine herbicides in urine, and compared with use of HLB sorbent [115]. MIPs have several advantages, for example physical robustness, rigidity, resistance to elevated temperature or pressure, and inertness toward organic solvents. The concentrator was constructed from a 2-mm capillary filled with MIP sorbent (particle size 55 μm) by use of a vacuum pump and then introduced into a 1.5-cm piece of PTFE tubing that fitted the outer diameter of the capillary. No frits were necessary to retain the sorbent. The results obtained for MIPs were superior to those for HLB sorbent.

Finally, during the last five years, increasing attention has been paid to the use of monoliths as sorbent in in-line SPE-CE. Monoliths are rapidly synthesized in one step and are characterized by low backpressure and chemical stability over a wide range of pH. Silica-based (prepared by use of sol–gel technology) and polymer-based (prepared by in-situ polymerization of monomers and cross-linkers) monoliths can be easily fixed at the end of a capillary by chemical modification [105, 116]. Several in-line SPE–CE applications with a variety of monolith materials have recently been proposed for analysis of, for example, methionine enkephalin in deproteinated cerebrospinal fluid [117], neurotransmitters (e.g., dopamine, adrenaline, histamine, and serotonin) in urine [118], or caffeine in urine [119].

Conclusions and future trends

Sample preparation is recognized as the most critical step in bioanalysis if good accuracy, selectivity, sensitivity, and robustness are to be achieved. Over the past few decades significant efforts have been devoted to reducing time, cost, manual handling, and consumption of solvents and samples. MEs have been shown to be very attractive compared with conventional LLE or SPE, and numerous innovative developments in respect of the liquid phase or miniaturized solid devices have been proposed. The combination of miniaturized sample preparation with CE has significant potential in bioanalysis, with only a few microliters of solvent required for the entire analytical process. Several MEs with CE analysis have been emphasized in this review, with a variety of bioapplications. A suitable approach should be selected considering the physicochemical properties of the analyte, the nature and volume of the biological matrix, the concentration range of the targeted analyte(s), the selectivity and sensitivity required, and the possibility of at-line, on-line and in-line automation.

Future developments will, hopefully, enable CE analysis to be used with the most recent sample pretreatments, which have already attracted attention in combination with LC or GC. As examples, disposable pipette extraction (DPX), in which a loose SPE sorbent is placed inside a pipette tip [120], was first proposed in 2008 and has already been successfully applied to a variety of applications with LC or GC analysis and is also fully adapted to CE analysis. In vivo SPME, in which sample preparation encompasses less-invasive sampling with direct exposure to human or animal living systems, could be of great interest in combination with CE for pre-clinical studies or clinical purposes [121]. Dried-blood spot sampling (DBS) has been shown to be not only a biofluid support but also a sample pretreatment with use of a small amount of solvents, inducing “on support” PP and selective desorption [122–124]. Moreover, recent microextraction techniques (e.g., SDME, HS-SDME, DLLME, and HF-LPME) substituting organic extraction solvents with non-toxic ionic liquids [125, 126] or natural oils [127] combined with CE could lead to powerful and solvent-free analytical procedures.

Surprisingly, especially in combination with liquid-based MEs, very few applications have revealed the potential of CE hyphenation with MS detection to substantially increase both sensitivity and selectivity. However, CE–MS is now easily implemented with dedicated interfaces, either with addition of a sheath liquid or in the sheathless configuration, and should undoubtedly be considered in combination with MEs for all bioanalytical applications to achieve the desired sensitivity (sub-ngmL^{-1} range) and provide the possibility of compound identification.

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Dispersive liquid–liquid microextraction combined with capillary electrophoresis and time-of-flight mass spectrometry for urine analysis

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ABSTRACT

The combination of dispersive liquid–liquid microextraction (DLLME) with capillary electrophoresis (CE) and a time-of-flight mass spectrometer (TOF-MS) was evaluated for the toxicological screening in urine samples. A methodology based on design of experiments (DOE) was implemented to increase the extraction efficiency. Dichloromethane and isopropanol were selected as the extraction and dispersing solvents, respectively. Seven factors for DLLME were screened with the help of a Plackett–Burmann DOE using two model compounds before fine investigation of the important parameters to maximise the compound extraction. These experiments were performed in the CE-UV configuration to overcome potential MS matrix effects. The performance of the entire procedure was then evaluated using CE-ESI-TOF-MS. With a preconcentration factor of more than 130, the highly sensitive DLLME-CE-ESI-TOF-MS method allowed for the detection of 30 toxicological compounds (*i.e.*, amphetamines and their derivatives, opiates, cocaine and its metabolites and pharmaceuticals) in urine with limits of detection in the *sub*-ng/mL level and was used to analyse real toxicological samples. The combination of DLLME and CE was particularly attractive because of the small amount of organic solvents required.

1. Introduction

Over the past two decades, greater attention has been paid to the use of chemical processes in an environmentally and human friendly way to suit green chemistry approaches. The latter goal consists of designing chemical processes to either reduce or eliminate hazardous substances, as guided by the 12 Principles of Green Chemistry [1]. In this context, new analytical procedures have been developed to protect people's health and to eliminate, or at least reduce, the negative impact of chemical products (*e.g.*, organic solvents) on the environment [1–3]. Much effort has been made to develop green analytical separation methods, especially with the advent of ultra-high performance liquid chromatography (UHPLC), capillary electrophoresis (CE) and supercritical fluid chromatography (SFC), which use significantly reduced quantities of organic solvents. For complex matrices or at very low analyte concentrations, the sample preparation step is considered to be the most polluting step of the analytical process [2,4]. Liquid–liquid extraction (LLE) and solid-phase extraction (SPE) are widely used for sample clean-up and analyte preconcentration; however, large volumes of hazardous organic solvents that are harmful to both humans and the environment are required for these extractions. Therefore, new sample preparation techniques have been developed over the last few years that (i) replace the toxic organic solvents used and (ii) reduce solvent consumption [4]. For the former, toxic solvents can be replaced with alternative, nontoxic extraction agents, such as supercritical fluids (supercritical fluid extraction, SFE), ionic liquids, superheated water (subcritical water extraction, SWE) and surfactants (cloud point extraction, CPE). For the latter, recent investigations have focused on developing miniaturised sample preparations that drastically reduce solvent consumption (*i.e.*, microextraction techniques) [5] or extract the analyte of interest without a solvent (*i.e.*, solventless sample preparation techniques) [6]. This miniaturisation can

Abbreviations: AEME, anhydroecgonine methyl ester; BGE, background electrolyte; CE, capillary electrophoresis; DCM, dichloromethane; DLLME, dispersive liquid–liquid microextraction; DOE, design of experiments; D-PX, D-propoxyphene; EDDP, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; EIE, extracted ion electropherogram; ESI, electrospray ionisation; EtOH, ethanol; EY, extraction yield; GC, gas chromatography; *i*-PrOH, isopropanol; LC, liquid chromatography; LLE, liquid–liquid extraction; LOD, limit of detection; *m/z*, mass-to-charge ratio; MA, methamphetamine; 6-MAM, 6-monoacetylmorphine; MBDB, N-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine; MDA, 3,4-methylenedioxyamphetamine; MDEA, 3,4-methylenedioxyethylamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; ME, matrix effect; MeOH, methanol; MPD, methylphenidate; MS, mass spectrometry; MTD, methadone; PE, process efficiency; RE, extraction recovery; SPE, solid-phase extraction; TOF, time-of-flight.

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also decrease the sample volume, analytical time, operating costs and both the discrimination and loss of compounds [5,7]. Both LLE and SPE can be miniaturised. Conventional SPE, which can be time-consuming, expensive, poorly reproducible because of differences in adsorbent batches and lacks selectivity versus matrix interferences, can be replaced with miniaturised SPE techniques, such as solid-phase microextraction (SPME), microextraction by packed sorbent (MEPS), stir bar sorptive extraction (SBSE) and disposable pipette extraction (DPX), with lower volumes required for the sample and extraction phase [7–9]. Because of the formation of an emulsion in LLE process and the relatively large amounts of both the sample and solvent required, as well as variable recoveries and difficult automation, liquid-phase microextraction (LPME) based techniques, such as single drop microextraction (SDME), hollow fibre LPME (HF-LPME), liquid–liquid–liquid microextraction (LLLME), directly suspended droplet microextraction (DSDME), drop-to-drop solvent microextraction (DDSME) and dispersive liquid–liquid microextraction (DLLME), tend to be used instead [7,8].

DLLME, first introduced in 2006, is based on a ternary solvent system in which a dispersive solvent allows for the dispersion of an extraction solvent into the sample [10–12]. The dispersing solvent must be fully miscible with both the aqueous sample and the extraction phase. Acetonitrile, acetone, isopropanol or methanol are usually selected as the dispersing solvent. The extraction solvent must be miscible with the dispersing phase but insoluble in water and must have a higher density. Chlorinated solvents such as chloroform, chlorobenzene, dichloromethane and tetrachloroethylene are conventionally used as extracting solvents [10,12]. A mixture of the extraction and dispersing solvents is rapidly injected into the aqueous sample with a syringe, which produces high turbulence that leads to the formation of tiny droplets. The surface area between the extraction solvent and the aqueous sample becomes very large, which allows for the rapid transfer of the analytes to the extraction phase and, therefore, reduces the extraction time. After centrifugation, the sedimented phase at the bottom of the tube is collected and either directly injected into the analytical system or evaporated to dryness before reconstitution and injection. Because of the properties of the extraction solvent, gas chromatography (GC) was the first analytical technique used to analyse the extracts for the determination of non-polar and volatile compounds, *e.g.*, pesticides or other contaminants in the water samples [10,11]. Secondly, DLLME was evaluated for the analysis of non-volatile compounds. These compounds are only suitable for GC analysis after a derivatisation step to increase their volatility. In this situation, the derivatisation was performed directly on the sample, which provided simultaneous derivatisation and extraction [13,14]. The combination of DLLME with liquid chromatography (LC), which is widely used for both qualitative and quantitative bioanalyses, has found increasing interest in recent years due to its applicability to a large number of compounds with different physicochemical properties [10]. Because halogenated solvents are incompatible with the mobile phases used in reversed-phase LC, an extra evaporation step is required before reconstituting in a compatible solvent and injecting. Recently, some DLLME-LC methods were investigated for pharmaceutical and toxicological compound analyses in biologically complex matrices, such as urine or serum [10,15,16]. Until now, only a few papers have described the use of DLLME in combination with CE and UV detection [17–23]. CE is a powerful separation technique with several advantages, such as low solvent and sample consumption (green analytical technique), short analysis time and high separation efficiency, especially for polar and ionised compounds. The combination of DLLME and CE is particularly attractive due to the low injection volumes required (only a few nL *versus* μL for LC analysis), which results in very high preconcentration factors.

In this study, the combination of DLLME with CE was evaluated for screening toxicological compounds in urine samples. For this purpose, the CE was coupled with a time-of-flight mass spectrometer (TOF-MS) using an electrospray ionisation source (ESI) and coaxial sheath-flow interface. Indeed, because the narrow optical path length afforded by the internal diameter of the capillary in the CE-UV configuration results in low sensitivity, a highly sensitive detector is required, especially for biological samples where the analyte concentration can be very low. In addition to its high sensitivity, TOF-MS also provides a high-speed data acquisition, which is particularly well-suited for the short peak widths obtained for CE separations, as well as a high mass resolution and accuracy, which are important for identification during the screening steps [24,25]. To obtain the best recoveries, a methodology based on a design of experiments (DOE) was implemented. A Plackett–Burmann DOE was used to evaluate the main effects of the extraction conditions on two model compounds (D-PX and MDMA) in CE-UV. The significant parameters were then investigated to maximise the extraction. Finally, the performance of the entire DLLME-CE-ESI-TOF-MS procedure was evaluated (*i.e.*, the matrix effect, extraction yield, extraction recovery and process efficiency) and eventually applied to both spiked urine and real toxicological samples.

2. Materials and methods

2.1. Chemicals and samples

2.1.1. Chemicals

Analytical grade isopropanol (*i*-PrOH), sodium hydroxide, hydrochloric acid, chlorobenzene and ammonia formate were purchased from Fluka (Buchs, Switzerland). Acetone and ethanol (EtOH) were obtained from Sigma–Aldrich (Seelze, Germany Steinheim, Germany). Chloroform and dichloromethane (DCM) were obtained from Acros Organics (Geel, Belgium). Methanol (MeOH), acetonitrile (MeCN) and formic acid, all of ULC/MS grade, were purchased from Biosolve (Valkenswaard, Netherlands). 3,6-Diacetylmorphine (heroine), codeine, norephedrine, meperidine (pethidine), 6-monoacetylmorphine (6-MAM), buprenorphine, ethylmorphine, amphetamine, methamphetamine (MA), 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyethylamphetamine (MDEA), N-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine (MBDB), ephedrine, pseudoephedrine, cocaine, methadone (MTD), 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and dextromethorphan in methanolic solutions (1 mg/mL) were obtained from Lipomed AG (Arlesheim, Switzerland). Cocaethylene and anhydroecgonine methyl ester (AEME) in acetonitrile (1 mg/mL) were also obtained from Lipomed. Nalbuphine hydrochloride hydrate, ketamine hydrochloride, procaine hydrochloride, lidocaine hydrochloride, trimipramine maleate, metoprolol and D-propoxyphene (D-PX) were purchased from Sigma–Aldrich (Seelze, Germany). Fentanyl citrate was obtained from Sintetica (Mendrisio, Switzerland). Methylphenidate (MPD) was a USP reference standard (Rockville, MD, USA). Ultrapure water was supplied by a Milli-Q RG purification unit from Millipore (Bedford, MA, USA).

2.1.2. Background electrolyte (BGE) and samples

The background electrolyte (BGE) consisted of a 20 mM ammonium formate buffer adjusted to pH 2.5 with formic acid. The pH value was measured using a SevenMulti pH meter (Mettler-Toledo, Schwerzenbach, Switzerland).

Six urine samples collected from healthy non-drug consumers were pooled directly after collection to obtain blank urine samples and stored in polypropylene tubes at −20 °C. The samples were

defrosted before sample preparation at ambient temperature and centrifuged at 10,000 rpm for 5 min (Heragus Biofuge 17 RS, Sepa-Tech, Engen, Germany). The supernatant was then filtered through a 0.45 μm Nylon filter (BGB Analytik AG, Böckten, Switzerland). Stock standard solutions of the solid analytes were prepared at 1000 mg/mL by dissolving each compound in MeOH and storing at 4 °C until use. Samples were prepared daily at the desired concentrations by evaporating the stock standard solutions to dryness under a gentle nitrogen steam (evaporator Techne DB 3D, Luzern, Switzerland) before reconstituting in blank urine.

Toxicological urine samples were received from the Laboratory of Clinical Chemistry (Geneva Hospital, Switzerland) and from the University Center of Legal Medicine (CURML, Geneva, Switzerland) and stored at −20 °C until use. Before sample preparation, these samples were defrosted at ambient temperature, centrifuged at 10,000 rpm for 5 min and filtered through a 0.45 μm Nylon filter.

2.2. Sample preparation

2.2.1. DLLME procedure

A 4 mL sample of urine was placed in a 15 mL polypropylene tube with a conical bottom, and 1 mL of 1 M NaOH was added to obtain the desired pH. A 1400 μL aliquot of isopropanol (*i*-PrOH) as the dispersing solvent containing 600 μL of dichloromethane (DCM) as the extraction solvent was rapidly injected (over two steps) with a 1 mL syringe (Hamilton, Reno, NV, USA) into the sample, which produced high turbulence. The solution was immediately centrifuged at 10,000 rpm for 5 min, and the organic phase, which had settled in the bottom of the tube, was collected with a syringe and transferred to a 1.5 mL polypropylene tube. Ten microlitres of an HCl–MeOH solution (1:99, v/v) was added before evaporating to dryness under a gentle nitrogen steam. The solid phase was reconstituted with 30 μL of a BGE–water mixture (1:9, v/v) before injection. The entire procedure was performed in an air-conditioned laboratory at 25 °C.

2.2.2. DLLME performance

The DLLME performance was evaluated in terms of the process efficiency (PE), matrix effect (ME), extraction recovery (RE) and extraction yield (EY) using a CE-ESI-TOF-MS configuration for a set of toxicological compounds according to the method previously developed by Matuszewski et al. [26] and completed by Marchi et al. [27]. A mixture containing amphetamine, ephedrine, MDMA, dextromethorphan, morphine, codeine, MTD and D-PX was used as the spiking solution. Four types of samples were required (Fig. 1). Sample A consisted of a mixture of the neat spiking solution in water as a standard. Sample B was the blank urine spiked with the spiking solution after extraction. Sample C was prepared from blank urine spiked before extraction. Sample D was a neat extraction standard consisting of water spiked before extraction. The concentration of the spiking solution in each sample was chosen to obtain a similar concentration after extraction by accounting for the dilution factor (133) and assuming a theoretical extraction recovery of 100%. The PE was calculated by comparing sample C to sample A. The ME was evaluated from the ratio of B to A. The RE was estimated from the ratio of C to B. Finally, the EY was assessed by comparing D to A. Each calculation was performed twice.

2.3. Instrumentation

2.3.1. CE-UV

CE-UV experiments were performed using an HP ^{3D}CE system from Agilent Technologies (Waldbronn, Germany) equipped with an on-capillary diode array detector, autosampler and power supply capable of delivering up to 30 kV. Separations were performed using a fused-capillary (BGB Analytik AG, Böckten, Switzerland) with a total length of 64.5 cm, an effective length of 56 cm and an internal diameter of 50 μm. Before its first use, the capillary was sequentially rinsed at 1 bar with MeOH (10 min), water (6 min), 1 M NaOH (10 min), water (6 min), 0.1 M HCl (10 min), water (6 min) and the BGE (10 min). The capillary was also preconditioned prior to each injection with fresh BGE at 1 bar (4 min). When not in use, the capillary was rinsed with water and then dry-stored. Samples were kept at ambient temperature in the autosampler and injected hydrodynamically at 50 mbar for 12 s (corresponding to 1.6% of the total capillary length) followed by a post-plug injection of the BGE at 50 mbar for 2 s (corresponding to 0.3% of the capillary length). Experiments were performed in the positive polarity mode (anode at the inlet) with a constant voltage of 30 kV and an initial ramping of 1667 V/s (18 s). The capillary was thermostated to 25 °C. UV/vis detection was carried out at 200 nm with a reference at 450 nm.

2.3.2. CE-ESI-TOF-MS

CE-ESI-TOF-MS experiments were performed using a G7100 CE system from Agilent Technologies. Separations were performed using a fused-silica capillary with a total length of 80 cm and an internal diameter of 50 μm. Before its first use, the capillary was rinsed at 2 bar with MeOH (5 min), water (3 min), 1 M NaOH (5 min), water (3 min), 0.1 M HCl (5 min), water (3 min) and the BGE (10 min) while keeping the ESI source open. Before each injection, the capillary was preconditioned with fresh BGE at 2 bar (3 min). When not in use, the capillary was rinsed with water and then dry-stored. Samples were kept at ambient temperature in the autosampler and injected hydrodynamically at 50 mbar for 25 s (corresponding to 1.7% of the total capillary length) followed by a post-plug injection of the BGE at 50 mbar for 2 s (corresponding to 0.1% of the capillary length). Experiments were performed in the positive polarity mode (anode at the inlet) with a constant voltage of 30 kV and an initial ramping of 1667 V/s (18 s). The capillary was thermostated to 25 °C. The CE instrument was coupled to a 6210 LC/MS TOF mass spectrometer from Agilent Technologies (Santa Clara, CA, USA) *via* a coaxial sheath-flow electrospray ionisation (ESI) interface from Agilent Technologies (Waldbronn, Germany). The sheath liquid was comprised of *i*-PrOH–water–formic acid (50:50:0.5, v/v/v) delivered at a flow rate of 3 μL/min by a 1200 isocratic pump from Agilent Technologies (Waldbronn, Germany). The drying gas temperature was set to 250 °C, while the drying gas flow rate and nebulising gas pressure were set to 4 L/min and 4 psi, respectively. These values were selected because they led to the best compromise of ionisation efficiency for a large set of low molecular weight basic compounds, including toxicological, forensic or pharmaceutical compounds. The ESI and fragmentor voltages were set to +4500 V and 150 V, respectively. The skimmer and first octopole voltages were set to 65 V and 250 V, respectively. MS detection was performed in the positive ion mode between the mass range of 50–1000 *m*/*z*, and 2.5 spectra/s were acquired (400 ms/spectrum, 5337 transients/spectrum). The automatic recalibration of each spectrum was achieved using purine (exact *m*/*z*: 119.036320) and the formate adduct of hexakis(tetrafluoropropoxy)phosphazine (exact *m*/*z*: 966.000725) as reference standards. Each $[M+H]^+$ was automatically extracted with a tolerance of ±0.005 Da to obtain the extracted ion electropherograms (EIE).

2.3.3. Software

CE Chemstation version A.10.02 (Agilent, Waldbronn, Germany) was used to control the CE-UV. CE Chemstation version B.04.02 (Agilent, Waldbronn, Germany) and MassHunter version B.03.02 (Agilent, Santa Clara, CA, USA) were used to control, respectively, the CE and ESI-TOF-MS data acquisition and handling. The BGE was prepared with the help of PHoEBuS software 1.3 (Analis, Namur, Belgium), and pK_a values were calculated using Advanced Chemistry Development (ACD/Labs) software version 11.02.

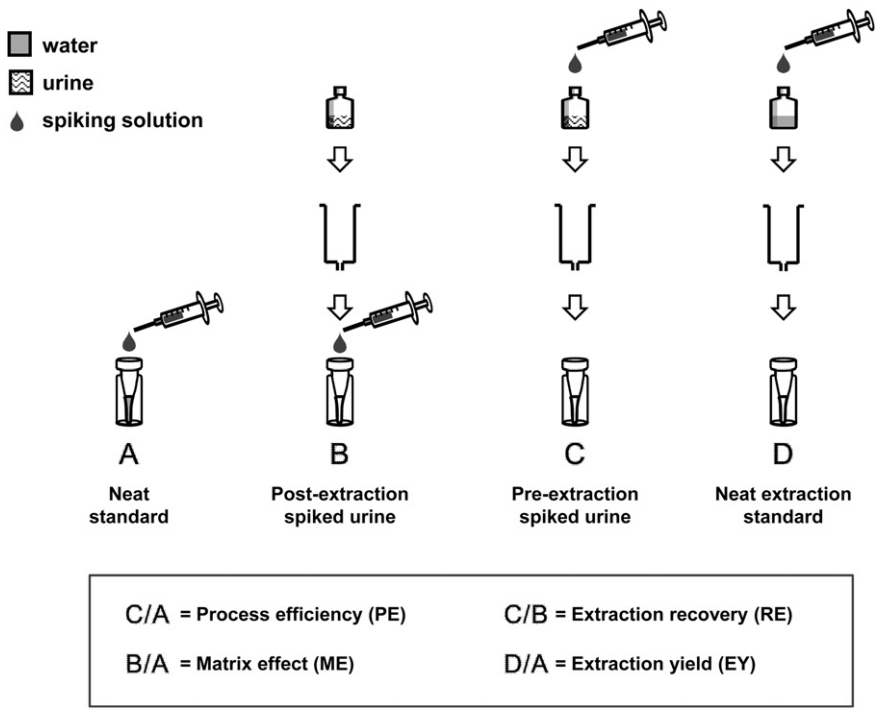

Fig. 1. Representation of the four samples required to evaluate the process efficiency (PE), matrix effect (ME), extraction recovery (RE) and extraction yield (EY). Adapted from [27].

3. Results and discussion

Because of the long detection time windows of toxicological compounds in urine (up to several days), a highly sensitive analytical method is required for screening to avoid false negatives. A sample pretreatment is particularly useful to preconcentrate the biological samples before analysis. Conventional liquid–liquid extraction (LLE) or solid-phase extraction (SPE) usually provide preconcentration factors between 10 and 100 and use a small volume of the sample (usually lower than mL range) to avoid using high volumes of organic solvents. The very low solvent consumption of DLLME allows for an increase in the treated sample volume and, consequently, the preconcentration factor. The development of the DLLME procedure was achieved using CE-UV to overcome any potential matrix effects that can be observed by ESI-MS detection. Two model compounds, MDMA and D-PX, were chosen for all investigations on the DLLME procedure, and lidocaine was selected as the internal standard for the CE injection correction.

3.1. DLLME development

The pH of the sample is particularly important for the extraction of ionisable compounds, such as toxicological compounds, that are basic with a pK_a between 6 and 10.5 (Table 1). As urines are, physiologically speaking, rather acidic, 1 mL of 1 M NaOH was added to the urine samples before their extraction to reach an adequate pH ($\geq$11.5).

3.1.1. Selection of the solvents

The selection of the dispersing and extraction solvents is of great importance for the DLLME procedure to obtain a good extraction. On the one hand, the extraction solvent should have a higher density than water, good extraction capacity for the target compounds and low miscibility in water. On the other hand, a high miscibility with the dispersing solvent is mandatory [10]. Three different halogenated solvents were investigated: chloroform (density 1.5 g/mL), dichloromethane (DCM, density 1.3 g/mL) and chlorobenzene (density 1.1 g/mL). One millilitre of isopropanol (*i*-PrOH) was used as a generic dispersing solvent and was mixed with 100 μL of the extraction solvent, except for DCM, where 200 μL was required to produce a sufficiently settled phase after centrifugation [15]. For each solvent, the process efficiency (PE), which represents the entire analytical process from sample preparation to detection, was assessed for both MDMA and D-PX (Table 2). DCM and chloroform gave rather good PEs whereas chlorobenzene had a very poor PE. DCM gave slightly better results than chloroform and was, therefore, chosen as the extraction solvent.

The dispersing solvent must be miscible in both the aqueous sample and the extraction solvent. Five dispersing solvents were tested in combination with DCM: methanol (MeOH), acetonitrile (MeCN), acetone, *i*-PrOH and ethanol (EtOH). Two hundred microlitres of DCM was mixed with 1 mL of each of the dispersing solvents before injecting into the sample. The PEs obtained for MDMA and D-PX are shown in Table 2. MeOH and EtOH were clearly poorly adapted because of their low PE. While the three other organic solvents all gave similar results, *i*-PrOH yielded the best PEs for both MDMA and D-PX and was selected as the dispersing solvent. In addition, *i*-PrOH gave the best phase separation after centrifugation.

3.1.2. Screening of relevant DLLME factors

As mentioned in previous studies [15,18], different factors can significantly influence the DLLME procedure, such as the volume of extraction and dispersing solvents, extraction time, centrifugation conditions, *etc.* A screening study was implemented to determine the most important factors involved in this procedure prior to further investigations. Seven factors were selected: the volume of 1 M NaOH used for pH adjustment (X_1), the volume of the solvent mixture (X_2), the ratio between the dispersing (*i*-PrOH) and extraction (DCM) solvent volumes (X_3), the presence of agitation (X_4), the extraction time (X_5), the centrifugation speed (X_6) and the centrifugation time (X_7). Because of the relatively high number of factors

Table 1
Physico-chemical properties of compounds of interest.

	Molecular weight (Da)	m/z of detected $[M+H]^+$ (±0.0050) (Da)	Basic pK_a [a] ± $IC_{95\%}$	Acidic pK_a [a] ± $IC_{95\%}$
Opiates				
Morphine	285.1365	286.1438	8.25 ± 0.40	9.48 ± 0.40
Codeine	299.1521	300.1594	8.23 ± 0.40	13.40 ± 0.20
6-MAM	327.1471	328.1543	8.03 ± 0.40	9.41 ± 0.40
Ethylmorphine	313.1678	314.1751	8.24 ± 0.40	13.40 ± 0.20
Fentanyl	336.2202	337.2274	8.92 ± 0.20	
Pethidine	247.1572	248.1645	7.84 ± 0.10	
Buprenorphine	467.3036	468.3108	8.31 ± 0.60	9.47 ± 0.60
Nalbuphine	357.1940	358.2013	7.35 ± 0.60	9.39 ± 0.60
Dextromethorphan	271.1936	272.2009	9.13 ± 0.20	
Methadone	309.2093	310.2165	9.05 ± 0.50	
EDDP	277.1830	278.1903	7.71 ± 0.60	
D-Propoxyphene	339.2198	340.2271	9.19 ± 0.28	
Amphetamines and their derivatives				
Amphetamine	135.1048	136.1121	9.94 ± 0.10	
MA	149.1204	150.1277	10.38 ± 0.10	
MDA	179.0946	180.1019	9.94 ± 0.10	
MDMA (ecstasy)	193.1103	194.1176	10.32 ± 0.10	
MDEA	207.1259	208.1332	10.34 ± 0.19	
MBDB	207.1259	208.1332	10.46 ± 0.20	
Ephedrine	165.1154	166.1226	9.38 ± 0.10	
Pseudoephedrine	165.1154	166.1226	9.38 ± 0.10	
Norephedrine	151.0997	152.1070	8.47 ± 0.10	
Methylphenidate	233.1416	234.1489	9.51 ± 0.10	
Cocaine and its metabolites				
Cocaine	303.1471	304.1543	8.97 ± 0.60	
Cocaethylene	317.1627	318.1700	9.04 ± 0.60	
AEME	181.1103	182.1176	7.97 ± 0.40	
Others				
Metoprolol	267.1834	268.1907	9.43 ± 0.10	
Procaine	236.1525	237.1598	9.24 ± 0.25	
Ketamine	237.0920	238.0993	6.46 ± 0.20	
Trimipramine	294.2096	295.2169	9.38 ± 0.28	

[a] Calculated using ACD/Labs software version 11.02.

with potential effects, a reduced factorial design was adopted. A Plackett–Burmann DOE was selected to determine the influence of these factors on the extraction. This DOE was applied for the screening of the seven parameters, with additional experiments at the central level to estimate the experimental variance. The investigated range for each factor is shown in Table 3. Lower (−1) and upper levels (+1) were determined by preliminary experiments and selected to obtain the widest range where a response was still observed. Because of the reduced number of experiments, the DOE was performed over a single day to eliminate any additional variability and block drift effects. PE was chosen as the analytical response for MDMA and D-PX.

The main effects were determined by the coefficient and the probability (p-value) calculated for each factor, as presented in Table 4, for MDMA and D-PX. The coefficients indicate the importance of each experimental factor, and a p-value $\leq |0.05|$ represents a significant effect on the PE, whereas $|0.05| < p$-value $\leq |0.10|$ indicates a relative effect on the PE. With regard to MDMA, two factors were considered to have a strong effect on the PE ($p \leq |0.05|$), the solvent mixture volume (X_2) and extraction time (X_5), which showed a positive and negative effect, respectively. Table 4 also reveals that, for MDMA, the centrifugation speed (X_6), centrifugation time (X_7) and agitation (X_4) were relatively important with p-values of 0.055, 0.054 and 0.106, respectively. The centrifugation

Table 3
Experimental factors (X) and levels involved in the Plackett–Burmann DOE.

Factors	Level		
	−1	0	+1
X_1 volume of 1 M NaOH (mL)	0.4	1	2
X_2 solvents mixture (mL)	1.0	1.5	2.0
X_3 $V_{extracting}/V_{dispersing}$ (%)	20	25	30
X_4 agitation (Y/N)[a]	N	Y	Y
X_5 extraction time (min)	0	5	10
X_6 centrifugation speed (rpm)	4000	7000	10,000
X_7 centrifugation time (min)	3	5	10

[a] Y = yes, N = no.

Table 2
Process efficiency (PE) obtained for MDMA and D-PX for evaluation of extraction and dispersing solvents ($n = 2$).

Solvent	PE (%)	
	MDMA	D-PX
DCM	44	40
Chlorobenzene	5	6
Chloroform	35	27
MeOH	4	4
MeCN	40	26
Acetone	35	49
i-PrOH	43	56
EtOH	16	12

Table 4
Coefficients and p-values obtained for MDMA and D-PX with Plackett–Burmann DOE.

Factors	MDMA		DPX	
	Coefficient	p-Value	Coefficient	p-Value
X_1 volume of 1 M NaOH (mL)	+0.021	+0.222	+0.002	+0.914
X_2 solvents mixture (mL)	+0.212	+0.024	+0.182	+0.044
X_3 $V_{extracting}/V_{dispersing}$ (%)	+0.021	+0.226	+0.023	+0.318
X_4 agitation (Y/N)[a]	−0.046	−0.106	−0.049	−0.161
X_5 extraction time (min)	−0.156	−0.032	−0.124	−0.064
X_6 centrifugation speed (rpm)	+0.091	+0.055	+0.102	+0.078
X_7 centrifugation time (min)	+0.092	+0.054	+0.069	+0.116

[a] Y = yes, N = no.

speed and time positively affected the PE, whereas agitation before centrifugation had a deleterious effect on the response. The volume of 1 M NaOH (X_1) and the ratio between the dispersing and extraction solvent volumes (X_3) did not significantly influence the PE. D-PX possessed similar tendencies, as shown in Table 4.

Complementary investigations were achieved for factors having a strong or relatively strong effect on the response, *i.e.*, the volume of solvent mixture (X_2), extraction time (X_5), the agitation (X_4) and both centrifugation speed and time (X_6 and X_7).

The volume of the solvent mixture (X_2) was investigated by injecting 1, 2, 3 and 4 mL into the sample. Above 3 mL, the extraction phase was formed on the top of the sampling tube; therefore, higher volumes were not considered. Extraction of the target analytes was enhanced with a 2 mL injection volume *versus* a 1 mL volume; 2 mL of the solvent mixture was thus injected into the samples to obtain the best PE. The total solvent volume required for DLLME was, therefore, very low, and only 500 μL was required for each mL of the urine samples, which is much lower than that required for a conventional LLE with a similar extraction performance.

The best analytical response was obtained with a 0 min extraction time (X_5). The decreasing performance with increasing time could be because of an equilibrium breakdown between the aqueous sample and both solvents. This extraction time is one of the greatest advantages of DLLME and indicates the process is immediate because of the large surface area produced during dispersion of the solvent into the sample [10].

The centrifugation speed (X_6), which positively affected the PE, was tested and fixed at the highest rate, 10,000 rpm. The centrifugation time (X_7) also positively affected the analytical response. Nevertheless, this parameter was tested and set to 5 min as a compromise between sufficient phase separation, good PE and time.

Several investigations were conducted both with and without sample agitation (X_4) after solvent mixture injection. According to the Plackett–Burmann DOE, this agitation had a deleterious effect on the PE. Therefore, samples were immediately centrifuged after injecting the solvent mixture without any agitation step.

Finally, the other operating parameter values were chosen according to their positive or negative influence on the PE. The ratio between the extraction and dispersing solvent volumes, which had a positive effect, was 30% DCM, which was the highest value that maintained a good dispersion in the sample, and 70% *i*-PrOH. The volume of 1 M NaOH added to the sample was set to 1 mL, which kept almost all of the toxicological compounds neutral in urine before extraction and enhanced the transfer to the organic phase.

3.2. DLLME performance

3.2.1. Preconcentration factor

As a relatively large volume (up to several millilitres) of urine can be easily and non-invasively collected, different urine sample volumes were investigated with the developed DLLME to enhance the preconcentration factor. The best compromise used 4 mL of urine and 2 mL of the solvent mixture.

The volume used for the reconstitution step before injection was reduced to further increase the preconcentration factor during sample preparation. In CE, even though very small quantities (nL range) are injected into the capillary, a minimum of 10 μL of solution is required in the injection vial because of equipment limitations. In this study, 30 μL was required for the final reconstitution to ensure injection repeatability.

Using 4 mL of urine and a final reconstituted volume of 30 μL resulted in a preconcentration factor of 133. This high preconcentration during the sample preparation step, assuming a theoretical process efficiency of 100%, increased the sensitivity of the analytical method considerably, which can be helpful for screening.

Table 5
Extraction performance expressed with matrix effect (ME), extraction yield (EY), extraction recovery (RE) and process efficiency (PE) for a representative set of toxicological compounds ($n = 2$).

	ME (%)	EY (%)	RE (%)	PE (%)
Amphetamine	100	101	76	76
MDMA	90	85	75	68
Ephedrine	92	67	61	57
Morphine	98	0	0	0
Codeine	95	88	74	70
Dextromethorphan	44	75	107	47
D-PX	40	68	104	42
MTD	35	74	90	32

3.2.2. Process efficiency, extraction recovery, extraction yield and matrix effect

The performance of the DLLME-CE-ESI-TOF-MS method was evaluated for four parameters, *i.e.*, the matrix effect (ME), extraction recovery (RE), extraction yield (EY) and process efficiency (PE), according to the method of Matuszewski et al. [26] and Marchi et al. [27] (Fig. 1). The ME, RE, EY and PE were calculated for a set of toxicological compounds representing commonly abused drugs. The results are found in Table 5.

MS signal alterations, such as signal enhancement or signal suppression, can be observed with CE-MS when analysing complex matrices, especially when using ESI, because of the so-called matrix effect. The ME has been known to either increase or decrease the MS signal because of interfering substances specific to the sample that comigrate with the targeted analytes and affect their ionisation. Sample pretreatment, such as LLE or SPE, greatly reduces the presence of these endogenous compounds; however, a small amount can remain in the sample, which leads to MEs [26–28]. These MEs can be evaluated qualitatively using a post-capillary infusion system as described for CE by Schappler et al. [29], which involved infusing a solution of the targeted compounds after the electrophoretic step while simultaneously injecting blank extracted samples. Matuszewski et al. proposed another strategy where ME can be quantitatively determined by comparing a neat standard to an extracted blank matrix spiked after extraction [26]. As shown in Table 5, a ME was measured for dextromethorphan, MTD and D-PX with calculated ratios between 35 and 44%. The remaining compounds did not demonstrate any signal alteration.

The EY represents the performance of the sample preparation itself for pure standards and is evaluated by comparing two water samples, *i.e.*, a neat standard and a neat extraction standard (water spiked before extraction) [27]. DLLME resulted in a good EY for all compounds except morphine. Besides its basic pK_a, morphine has an acidic function which was completely ionised at the pH of the samples (*cf.* Table 1).

The RE expresses the EY for the DLLME when applied to the matrix. Indeed, both endogenous interferents and the matrix itself affect the extraction with endogenous interferents usually lowering the RE, and urine samples increasing it because of the inherent salting-out effect [30]. The latter could explain why the REs obtained for matrix samples were better than the EY for water samples for both dextromethorphan (107% *versus* 75%) and D-PX (104% *versus* 68%). No other significant differences were observed when comparing the RE to the EY for the analytes (Table 5).

Finally, the PE, which is a combination of the RE and ME and corresponds to the absolute performance of the entire analytical process, was quite good (≥50%) for most of the compounds not suffering from matrix effects or poor extraction recoveries.

It has to be noted that in conventional LLE, the extraction can be repeated to increase the analyte recovery. This strategy was also investigated with the DLLME procedure, but a second extraction was impossible because a small amount of dispersing solvent

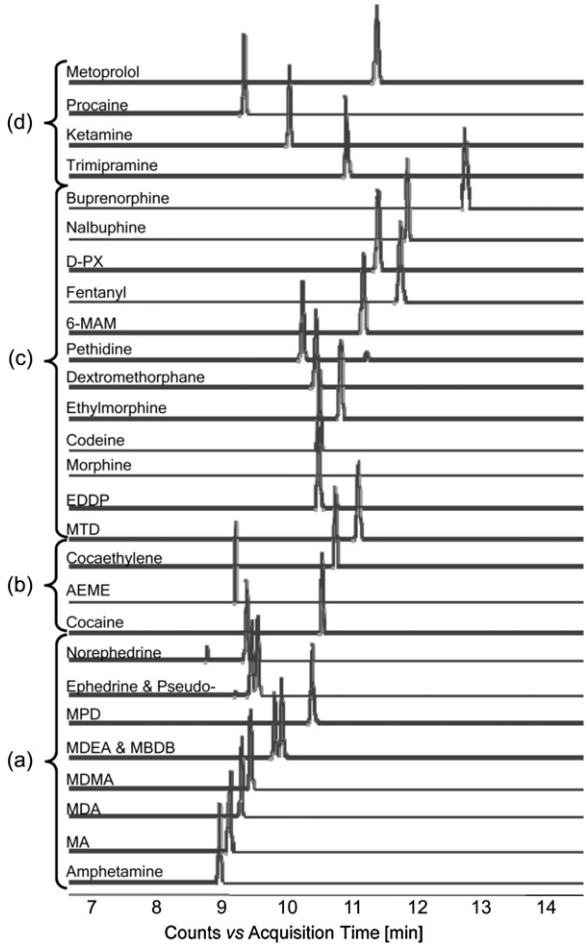

Fig. 2. Extracted ion electropherograms (±0.005 Da) of blank urine spiked with a set of forensic and pharmaceutical compounds at 10 ng/mL, extracted with DLLME and analysed by CE-ESI-TOF-MS. (a) Amphetamines and their derivatives, (b) cocaine and its metabolites, (c) opiates, and (d) various toxicological and pharmaceutical compounds. See Sections 2.2.1 and 2.3.2 for the extraction and analytical conditions.

remained dispersed in the aqueous sample after the first organic phase collection. When the sample was extracted again, a change in ratio between dispersing and extraction solvents occurred, leading to very bad dispersion and a lack of separation phase after centrifugation.

3.3. Application to real samples

Blank pooled urine was spiked to 10 ng/mL of almost 30 representative drugs of abuse and their main phase I metabolites, *e.g.*, cocaine and its derivative, opiates, amphetamine and its derivatives and some pharmaceuticals (Table 1). Urine was then extracted *via* the optimised DLLME procedure and analysed by CE-ESI-TOF-MS. The obtained electropherograms for each compound are shown in Fig. 2. All of the analytes were separated and detected with the exception of morphine, which was not extracted, as previously mentioned. The powerful combination of the DLLME sample pretreatment and CE-ESI-TOF-MS provided high sensitivity with limits of detection (LODs), expressed as the signal height above 500 counts, below the ng/mL range for most compounds (Table 6). Amphetamines and almost all of its derivatives had LODs from 0.25 to 0.50 ng/mL. Opiates were detected at concentrations as little as 0.10 ng/mL. However, the LODs for cocaine and its metabolites were slightly higher, but still under 10 ng/mL.

Urine samples collected from drug users were extracted using the DLLME procedure and analysed. Compound identification was performed by comparing the migration times to those of the spiked blank urine standards and by accurate mass determination. The electropherograms of two urine samples are shown in Fig. 3. The first sample contained cocaine and two of its metabolites, AEME and cocaethylene (Fig. 3a). The detection of these phase I metabolites

Table 6
Limits of detection (signal heights above 500 counts).

	LOD (ng/mL)
Opiates	
Morphine	–
Codeine	0.50
6-MAM	5
Ethylmorphine	0.50
Fentanyl	0.25
Pethidine	0.10
Buprenorphine	1
Nalbuphine	10
Dextromethorphan	0.25
Methadone	0.25
EDDP	0.25
D-Propoxyphene	0.25
Amphetamines and their derivatives	
Amphetamine	0.25
MA	0.25
MDA	0.50
MDMA (ecstasy)	0.25
MDEA	0.25
MBDB	0.25
Ephedrine	0.25
Pseudoephedrine	0.25
Norephedrine	1
Methylphenidate	0.50
Cocaine and its metabolites	
Cocaine	5
Cocaethylene	5
AEME	10
Others	
Metoprolol	0.50
Procaine	0.25
Ketamine	0.25
Trimipramine	0.50

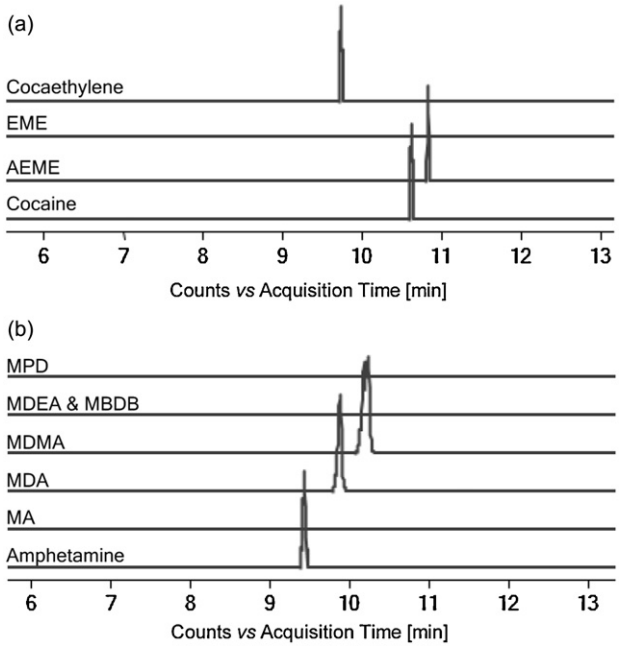

Fig. 3. Extracted ion electropherograms (±0.005 Da) obtained from real toxicological urine samples extracted by DLLME prior to CE-ESI-TOF-MS analysis. (a) Sample #1: cocaine and its metabolites and (b) sample #2: amphetamines and their derivatives. See Sections 2.2.1 and 2.3.2 for the extraction and analytical conditions.

was of great interest for providing complementary information beyond the presence of main compound cocaine. Indeed, AEME is a pyrolysis product that can only be detected from smoked cocaine (*crack*), whereas cocaethylene is produced by the concomitant consumption of cocaine and ethanol from the transesterification of the cocaine by the ethanol. For the second urine sample, amphetamine, MDMA (ecstasy) and MDA were detected (Fig. 3b). The detection of MDA was informative because it is an active metabolite obtained after the N-demethylation of MDMA. The primary compounds extracted and detected by DLLME-CE-ESI-TOF-MS from the toxicological urine samples were confirmed by immunoassay; however, more information was obtained from the phase I metabolites detected by the developed analytical method because of its high selectivity and sensitivity. The combination of a miniaturised sample preparation, such as DLLME, with a powerful separation and detection technique allowed for the rapid and efficient screening of real toxicological urine samples with low solvent consumption.

4. Conclusion

The DLLME combined with CE-ESI-TOF-MS was evaluated for the toxicological screening of both polar and basic compounds in urine samples. DCM and *i*-PrOH were selected as the extraction and dispersing solvents, respectively. Seven operating parameters of the DLLME procedure were first screened with the help of a Plackett–Burmann DOE in CE-UV configuration prior to the fine investigations on the significant parameters to maximise compound extraction. The performance of DLLME with a CE-ESI-TOF-MS configuration was studied for a set of representative toxicological compounds. The ME, EY, RE and PE were determined and emphasised different extraction and ionisation behaviours based on the class of compounds.

A small volume of organic solvent was required for the sample preparation with less than 500 μL used per millilitre of urine. To increase the sensitivity of this method, 4 mL of urine sample was extracted and evaporated before a final reconstitution in a very small volume. For compounds which presented good PE (>50%), the preconcentration factor was more than 75 and could reach 100. The high sensitivity of the developed analytical method allowed for the detection of almost thirty toxicological compounds in urine with limits of detection in the *sub*-ng/mL level. DLLME-CE-ESI-TOF-MS could be applied to detecting toxicological compounds and phase I metabolites in real samples.

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Chapter III.

Chapter III. Multi-target screening and quantitation by CE-MS

1 Introduction

As already stated in **Chapter I**, the combination of CE with MS presents some advantages that are particularly relevant in clinical and forensic toxicology, such as the low sample consumption and the speed of analysis, as well as an orthogonal principle of separation to chromatographic methods, which is for example useful for confirmation purposes. Up to now, few studies were performed to evaluate the potential of CE-MS in these fields, whether for a general unknown screening or for (multi-)target analysis. This may be due to a variety of reasons, which are herein presented.

Although much higher than in CE-UV configuration, the sensitivity provided by CE-MS is often still insufficient due to the very low sample volumes conventionally introduced. **Chapter II** provided some relevant strategies to increase the overall sensitivity by providing important preconcentration factors during the sample preparation. Considering the whole CE-MS process, the sensitivity can be further increased at the following stages: (i) injection, (ii) ionization, and (iii) ion transmission.

Only few nL of the sample are generally introduced into the capillary with the conventional HD injection. The volume of injection depends on the length of the capillary; *i.e.*, the sample zone length should be less than 1-2% of the total capillary length to maintain good efficiency. For an 80-cm capillary length and 50-µm i.d. (standard CE-MS capillaries), this represents 16 to 32 nL of sample injected. Higher injected volumes lead to peak broadening. Moreover, mismatched conductivity between the BGE and a large sample zone can lead to inhomogeneous electric field and perturbation during the analysis or bad peak shapes. The loaded quantity can be strongly increased in HD injection without peak broadening by using on-line sample preconcentration techniques, referred to as *sample stacking* methods. Sample stacking is performed directly in the separation capillary and is based on the velocity change of the analytes due to field strength or pH differences between the sample zone and the running buffer. EK injection, where the analytes enter in the capillary under the application of an electric field, may be also envisaged to enhance the loaded quantity. However, EK injection leads to a discrimination of the analytes injected according to their effective mobility and is not really adapted to samples presenting variable conductivities (*e.g.*, urine), providing lower injection repeatability than HD injection. Numerous sample stacking procedures can be implemented depending on the composition of the sample, as hereafter discussed.

Multiple source parameters can influence the gas-phase ions production in CE-ESI-MS, according to the configuration of the interface. In sheath-flow configuration, the electrospray process depends on the sheath-liquid composition and its flow rate, the sprayer shape, the needle material (stainless steel, Pt) and its tip shape, the position of the capillary and its tip cut, the geometry of the source, and the source position and distance from the MS entrance. Some of these parameters can be modified by the operator but the sprayer shape and the source geometry are defined by the manufacturer. In this context, a modified ESI source (developed for LC-MS purposes but also used for CE-MS hyphenation) and a revised design of the CE-ESI-MS sprayer have been recently proposed by Agilent Technologies to improve the desolvation process and increase the ionization efficiency by a

spatial focusing of ions. Both innovations have been studied during this work to evaluate the potential gain in sensitivity.

Once the gas-phase ions obtained at atmospheric pressure, they enter into the MS and pass through sequential pumped vacuum chambers until they reach the high vacuum region of the mass analyzer and, finally, the detector. The ion transmission efficiency, *i.e.,* the fraction of the gas-phase ions produced by electrospray process that enters the MS entrance and reaches the detector, is typically very low, estimated on conventional MS instruments at 10-1000 ppm of the produced ions due to losses at the MS inlet and at the skimmer [1]. Therefore, numerous attempts in enhancing the overall ion transmission have been carried out over the last years by MS manufacturers to improve the sensitivity, for example by replacing the skimmer by ion funnels which ensure a better transmission between the first and second vacuum stages [2].

Besides these sensitivity issues, CE-MS is also often considered less repeatable than chromatographic methods. The lower repeatability concerns both injection process and migration times. HD injection is performed by applying a small pressure, typically 30-50 mbar, during few seconds. Due to instrumental limitations, this process can lead to rather high variabilities in the injected volume, particularly when using short capillaries. Simply placing the capillary within the vial already creates an injection due to the capillary action; however, this effect is most frequently not significant. In CE-MS configuration, additional injection variability is observed due to the presence of the ESI source; the latter should always be turned on only after the sample has been introduced to avoid any effect caused by the nebulizing gas on the injection process. An IS correction is always recommended in case of compounds quantitation, not only to overcome any potential matrix effects or losses during the sample preparation, but also to correct the variability encountered during the injection process. The low migration times' repeatability is a known phenomenon in CE that may be observed within the same capillary or between capillaries. Indeed, using bare-fused capillaries without particular attention will lead to large shifts in migration times depending on the analyzed matrix, even up to several minutes. The inner surface of the capillary is not chemically inert and can interact with both analytes and matrix constituents. This is particularly true in case of complex matrices or with protein analysis, as discussed in **Chapter IV**. Working at pH values between 4 and 7 should be avoided due an unrepeatable EOF. In order to decrease the potential solute-surface interactions, the use of low or high pH values, and high buffer ionic strength is recommended, as well as rinsing procedures between runs. New capillaries can present some impurities when purchased and variable residual silanol concentrations; rigorous capillary conditioning is thus strongly recommended, as well as in daily routine. When not in use, capillaries should be dry-stored to avoid any contamination or clogging. If all these recommendations are not sufficient, a chemical wall modification can be envisaged by coating the inner surface of the capillary. With coated capillaries (in-house or commercially available), largely acceptable repeatabilities are obtained by preventing solute-surface interactions, even with the analysis of complex matrices. Thermostating the capillary is also important to avoid any migration time's shifts due to an increased temperature, even with coated capillaries. This is particularly challenging for the part of the capillary positioned out of the CE apparatus, thus prone to temperature variations. The latter are less encountered in air-conditioned laboratories [3].

With the emergence of fast or ultra-fast technologies such ultra-fast GC-MS or fast (UHP)LC-MS, CE analysis is not really considered *high-speed* anymore. Nevertheless, in a view of the whole analytical process, CE remains competitive. Even with an analysis in less than 1 minute, ultra-fast GC still requires a time-consuming hydrolysis and/or derivatization step prior to the injection. Compared to LC and GC, a sample dilution is frequently sufficient prior to CE injection, even for rather complex matrices, provided that the injection is repeatable and between-runs rinsing procedures implemented. If an extraction procedure is required, CE may allow for a direct injection of the organic extract without any evaporation and reconstitution step. In HPLC, a re-equilibration of the column after each run is required, while in CE the capillary can be simply flushed during 2 or 3 min with BGE, corresponding to the re-equilibration time occurring in UHPLC. In order to speed the CE separation itself, a basic BGE can be selected to increase the EOF towards the MS or use anionic coated capillaries which provide high and repeatable EOF. With all these strategies rounded up, a complete analysis can be performed in less than 10 to 15 min.

Finally, implementing a CE-MS procedure for general unknown screening of basic, neutral, and acidic compounds is rather challenging. The separation principle of CZE does not allow for the discrimination of compounds not baring a charge at the selected pH. Other CE modes such as MEKC can yet be envisaged but are not easily combined with MS. Moreover, the composition of the sheath liquid is rarely generic, containing either formic or acetic acid, or ammonia. An alternative strategy can be implemented combining (i) a BGE presenting an intermediate pH, (ii) an anionic coated capillary to ensure the migration times repeatability at the selected pH and provide a high EOF towards the MS, (iii) a sheath liquid containing both acidic and basic electrolytes, and (iv) the use of ESI-MS polarity switching or time segments. This is illustrated in an article presented in **Appendix III**, where melamine and its related compounds ammeline, ammelide, and cyanuric acid were analyzed by CE-ESI-MS within a single run with a BGE composed of 25 mM ammonium acetate at pH 5.2 and a sheath liquid consisting in a mixture of isopropanol:water:ammonia, 50:50:2 (*v/v/v*) containing 25 mM acetic acid. Ammonia had the ability to deprotonate acidic analytes while providing a proton to basic compounds. Acetic acid was added to stabilize CE and ESI currents and reduce baseline fluctuations. After the detection in ESI positive mode of melamine and ammeline, migrating under cationic state, the ESI polarity was switched to ESI negative and ammelide and cyanuric acid, both migrating under anionic form, were detected. This strategy was made possible with the mass analyzer used (*i.e.*, single quadrupole) due to the limited number of analytes of interest.

The large majority of drugs of abuse or compounds of forensic/clinical interest present basic properties. Thus, a CE-MS screening procedure can be implemented with operating conditions adapted to the analysis of basic compounds. This approach was selected in this work for the implementation of a multi-target screening procedure of drugs of abuse in urine samples followed by their quantitation.

2 Improvements in CE-MS performance

Considering all the aspects discussed in the last Section, a *high performance* CE-MS workflow was developed for the screening and quantitation of drugs of abuse in urine and is presented in **Article III**. Attempts in enhancing the performance were carried out by simplifying the sample pre-treatment,

implementing an on-line sample preconcentration, using a new ESI configuration, as well as high resolution or high sensitive mass analyzers. All these approaches are discussed hereafter.

2.1 On-line sample preconcentration

2.1.1 Principles

On-line sample preconcentration by stacking approach is performed directly in the separation capillary and allows for the injection of 5 to 70 % of the capillary length without peak broadening by focusing the analytes into a minimal volume. Sample stacking is based on the electrophoretic velocity change of the analytes due to differences in the field strength or chemical composition (*e.g.,* pH, addition of micelles, *etc.*) between the sample and the BGE, resulting in the concentration of the analytes in a narrow zone at their boundary [4-7]. Stacking procedures can be categorized into two groups based on the phenomenon for concentrating the analytes, *i.e.*, techniques with field-strength induced changes, where the velocity of the analytes is modified, and techniques with chemically induced changes, where the chemical composition of the sample is modified [8-10].

Basically, stacking phenomenon relies on the principle of concentration adjustment derived from a conservation law, the Kohlrausch regulating function (KRF). For monohydric strong and weak univalent acids and bases, KRF is expressed by **Eq. 3.1**:

$$KRF(x) = \sum_{i=1}^{N} \frac{c_i(x,t)}{\mu_i} \qquad \text{(Eq. 3.1)}$$

When considering a 1D movement along the x axis, the total analytical concentration c_i of the i^{th} ion is a function of the position x and the time t, and μ_i is the absolute mobility of ion i. The KRF is constant at any position x of the capillary at a time t, meaning that when an analyte migrates from a region with a low KRF (*e.g.*, sample zone) into a region with a higher value of KRF (*e.g.*, BGE), its concentration is automatically adjusted (*i.e.,* increased) when it passes the boundary to keep the KRF constant [11,12].

2.1.2 Field-strength induced changes

Stacking approaches based on field-strength induced changes involve the velocity change of the analytes between the sample zone and the BGE caused by the modification of the electric field strength. The most relevant techniques are the field-amplified sample stacking (FASS), the field-amplified stacking injection (FASI), the large-volume sample stacking (LVSS), and the isotachophoretic stacking (transient isotachophoresis, tITP).

2.1.2.1 Field-amplified sample stacking and field-amplified stacking injection

FASS (referred to as *field enhanced sample stacking* by Simpson *et al.*, finding that *amplified* is not a philological term for an electric field [6]), used with HD injection, is achieved with a sample having a lower conductivity than the BGE. Applying the separation voltage results in different electric field

strengths for the sample and the BGE. The sample analytes migrate rapidly until they reach the BGE. Exposed to the lower local electric field strength, the velocity abruptly decreases, leading to a simultaneous local concentration increase and a shortening of the analyte zone. Analytes stack in a narrow zone at the boundary and then begin the separation. If the analyte is dissolved in a diluted BGE, the field enhancement factor γ is expressed by **Eq. 3.2** [13]:

$$\gamma = \frac{E_1}{E_2} = \frac{\kappa_2}{\kappa_1} = \frac{c_2}{c_1} \quad \text{(Eq. 3.2)}$$

Where E_1 and E_2 are the electric field strength of sample and BGE, respectively, κ the electric conductivities in the two compartments, and c_2 and c_1 the concentrations of analyte in BGE and sample, respectively.

Therefore, the sample has to present a lower conductivity than the BGE, *e.g.*, by diluting it with water or organic solvents, or by desalting. FASS is thus not really adapted to biological matrices. It is also limited to samples that occupy less than 5 % of the capillary volume. Higher amounts will lead to peak broadening due to an electroosmotic velocity greater in the sample than in the BGE, causing a hydrodynamic flow between the two zones; or, in case of low EOF, due to molecular diffusions arising from elongated migration times occurring with longer sample plug [10,14].

FASI (or FESI, for *field enhanced sample injection*) involves the same principle but the sample is electrokinetically injected. A short water plug is frequently injected before the sample to enhance the stacking phenomenon, especially for moderate conductive matrices. Larger sensitivity enhancements can be obtained with FASI compared to FASS. However, due to the principle of injection, analytes are injected to a different extent based on their mobility, thus causing a bias in favor of high-mobility ions. The value and direction of EOF has also to be monitored, as in conventional EK injection [4,6].

2.1.2.2 Large volume sample stacking

In order to inject more than 5 % of the capillary in FASS without band broadening, LVSS can be implemented. LVSS involves the sample stacking by continuously removing (with external pressure or EOF) the sample matrix from the capillary. The direction of pumping is opposite to the migration of the analytes and at a lower velocity. The matrix removal is stopped before the analytes exit the capillary by polarity switch or variation of EOF. The polarity switching strategies is most commonly used and involves the injection of a low-conductivity sample matrix, as for FASS, but for a longer period of time. A voltage is then applied, leading to the stacking phenomenon, as well as an external pressure to remove the matrix in the direction of the inlet. Once the current has reached 70-95 % of the current value observed when the capillary is filled with 100 % BGE, the matrix removal is stopped by polarity switching, and the separation begins [5,10]. LVSS is well adapted for anions stacking but requires coated capillaries for cationic compounds.

2.1.2.3 Isotachophoretic techniques

Isotachophoresis (ITP) is a CE mode where the separation of the compounds occurs in a discontinuous running buffer. This principle is used in CZE as a stacking procedure, thereby referred to as *transient isotachophoresis* (tITP). tITP relies on the injection of the sample between a leading ion (*leading electrolyte*, LE) and a terminating ion (*terminating electrolyte*, TE) whose mobilities are greater and lower than any analyte ion, respectively. By applying the separation voltage, an electric field gradient is established due to the different zone conductivities leading to the analytes stacking behind the LE, as a function of their effective mobility and the KRF [9,15]. After the sample focusing, the BGE co-ions start to enter in the stacked sample zone thus destroying the ITP composition. This diluted zone can then begin the separation. On the contrary of FASS or FASI, tITP is well adapted to biological samples with high salt content; both chloride and sodium ions can act as LE due to their greater mobility and concentration than organic ionic compounds present in the sample, since a concentration of LE at least 50 times higher than the analytes of interest is required to ensure the stacking. Moreover, tITP can be easily coupled with MS detection, provided the use of volatile LE and TE, such as ammonium acetate.

Numerous tITP set-ups exist, depending on the nature of LE and TE, if the BGE acts as LE, or if the sample acts as TE or LE. Another example is the use of tITP with addition of low-conductivity organic solvent in the sample, referred to as *pseudo-ITP* [4,15,16]. Typically, this can occur with serum or plasma sample after acetonitrile deproteinization. While NaCl acts as LE, acetonitrile performs the role of TE by lowering the conductivity of the sample matrix, thus leading to high electric field strength and zone sharpening. This pseudo-ITP stacking effect has also been observed with other low conductivity alcohols. Although well adapted to urine or blood samples and providing interesting preconcentration, pseudo-ITP remains sparsely used.

2.1.3 Chemically induced changes

Besides preconcentration procedures based on differences in electric fields, modifications of the composition of the sample can also lead to analytes stacking. Addition of pseudo-stationary phases such as micelles or using a change in pH produce a modification in the electrophoretic mobility which can be used to focus the analytes.

2.1.3.1 Sweeping

Sweeping involves the interaction of a pseudo-stationary phase present in the BGE and a sample with similar conductivity but free of the pseudo-stationary phase. The pseudo-stationary phase is generally composed of micelles and the interactions rely on the same principle than MEKC separation mode, *i.e.,* chromatographic partitioning and electrostatic interactions. Sweeping can be used to increase the loading quantity of both neutral and charged compounds. CE polarity and charge of the micelles are selected to allow for their migration through the sample zone by applying the separation voltage. When the micelles penetrate the sample zone, they “sweep” the analytes, picking them up and accumulating them in narrow zones. The effectiveness of the stacking depends on the affinity of the analyte for the pseudo-stationary phase, as expressed by **Eq. 3.3**:

(Eq. 3.3)

$$l_{sweep} = l_{inj}\left[\frac{1}{1+k}\right]$$

Where l_{sweep} is the length of the analyte zone after the sweeping, l_{inj} the length of the sample solution injected, and k the retention factor [6,8,17]. In case of different conductivities between sample and BGE, sweeping can also be performed but additional mechanisms are thus involved and may affect the focusing result of sweeping (positively for compounds with low k). Different additives forming micelles or other pseudo-stationary phases have been already successfully tested, such as SDS, cyclodextrines, EDTA, microemulsions, or ILs [10]. Due to its universality (application to both neutral and charged compounds), sweeping is widely used in various fields. Other techniques based on preconcentration with micelles have been also recently proposed, such as the *analyte focusing by micelle collapse* or the *micelle to solvent stacking* [9]. However, sweeping is not easily combined to MS detection to the presence of the micelles.

2.1.3.2 Dynamic pH junction

Dynamic pH junction preconcentration technique is based on the pH differences between the BGE and the sample and was first proposed by Britz-McKibbin *et al.* [18] based on preliminary work of Aebersold and Morrison [19]. Dynamic pH junction is particularly useful for weak anionic or basic compounds, which present different ionization states depending on the pH, leading to changes in velocity. A substantial difference in mobility of the analyte at the two different pH values is required to observe a significant change in velocity. As an example of dynamic pH junction, a weak acidic analyte present in an urine (acidic) sample is injected as a long plug in a capillary filled with an alkaline BGE. When applying the positive separation voltage, the hydroxide ions from the BGE migrate to the acidic sample which is thus gradually titrated. The acidic analyte will thereby become ionized in this zone and migrate to the anode (inlet). If it enters in the acidic sample zone, it will become neutral again, and stop the migration. Not only the difference in pH between the sample and the BGE is important, but also the composition of both parts, having a strong influence on the speed and duration of the stacking process due to additional stacking mechanisms such as tITP [6,20,21].

2.1.4 pH-mediated stacking

Depending on the authors, the pH-mediated stacking, also referred to as *moving chemical reaction boundary*, is categorized in either field-induced stacking or chemically-induced stacking procedures. Basically, both mechanisms are involved and are interdependent, as for the dynamic pH junction where a discontinuity in the pH produces differences in field strength. pH-mediated stacking was developed by Lunte and co-workers to allow for the application of FASS to high-conductivity matrices by titrating the injected zone to neutrality, thus creating a low-conductivity region; and is thus well suited for biological matrices [22,23]. Only one additional step is required, *i.e.*, the injection of a strong base or acid after the sample. For the stacking of cationic compounds, the sample is electrokinetically introduced in the capillary while the BGE anions (*e.g.,* acetate) migrates in the opposite direction and displace the anions of the sample. A plug of strong acid (*e.g.*, formic acid) is then electrokinetically injected. The protons H^+ from the acidic plug migrate quickly through the sample zone, titrate the acetate anions, leading to a region of neutral charge and, thus, of low conductivity. Field amplification occurs in this zone with stacking of cationic analytes [8,11,24]. The

procedure is the same for anionic compounds but with reversed EOF and reversed separation polarity, as well as the injection of a strong alkaline plug [25].

Not only EK injection can be used in pH-mediated stacking, as stated by Arnett and Lunte who tested the use of HD injection for either the sample (*HD/EK mode*) or the post-plug (*EK/HD mode*) injection, or even both (*HD/HD mode*). The stacking was found the least efficient when using HD/HD mode, leading to poor peak shape, and explained by a lower concentration of titrating ions in the sample [25].

The HD/HD mode was also used by Neusüss *et al*. who proposed a pH-mediated stacking for the stacking of peptides with an additional basic pre-plug [26]. The capillary was first hydrodynamically filled with 1-2 M NH_4OH (1 % of the capillary length) prior to the HD injection of the acidic sample (10 % of the capillary length), and subsequent HD injection of 4 M formic acid (1 % of the capillary length). By applying the separation voltage, the protonated peptides migrated in the sample zone until they reached the strong alkaline plug and became neutral. The ammonia plug was then acidified by the sample and the running buffer, and the peptides became charged again and began the separation. The stacking allowed combined with CE-MS/MS and sheath-flow interface allowed for LODs in the low nM range. This approach of pH-mediated stacking with a preplug of ammonia and a post-plug of formic acid was also used for the determination of cationic metabolites extracted from the bacterium *D. vulgaris* by CE-FT-ICR/MS [27].

Finally, the use of this NH_4OH pre-plug prior to injection of acidified sample but without an acidic post-plug was proposed by Mayboroda and Neusüss for the aminoacids profiling in urine [28], and subsequently selected by Wang *et al.* [29], Ramautar *et al.* [30], Nevedomskaya *et al.* [31], Yu *et al.* [32], and Tak *et al.* [33] for the stacking of amino acids or metabolites in urine. All these studies were performed in CE-MS with concentration of NH_4OH ranging from 12.5 to 20 %, sample diluted with an acidic BGE, and a volume of sample injected corresponding to 10 to 20 % of the capillary length, which led to significant increase in sensitivity.

This approach of pH-mediated stacking was selected in this study for the preconcentration of drugs of abuse during both screening and quantitative step. Urine samples were first diluted with water and BGE (1:8:1) to normalize the pH of all urine samples (pH ≤ 2.5), acidify the sample for a proper stacking, and lower the urinary salts concentration. The BGE consisted of 1 M formic acid at pH 1.8, which allowed for a complete ionization of the large majority of drugs of abuse. A relatively high ionic strength was found advantageous for the stacking. The injection parameters were investigated, *i.e.*, concentration of NH_4OH as well as pre-plug and injection plug length. The most efficient stacking was obtained with a pre-plug of 7 % NH_4OH corresponding to 0.7 % of the capillary length, followed by the injection of the acidified urine corresponding to 20.5 % of the capillary length, and a post-plug of BGE corresponding to 0.2 % of the capillary length. The mechanism occurring during the stacking is proposed in **Fig. 3.1**. Higher loading volumes were tested, but instability of the capillary coating material and of the CE-MS analysis were observed at loading volumes corresponding to more than 20.5 % of the capillary length. Moreover, increasing the concentration or plug length of NH_4OH led to an enhanced ion suppression, observed with a decrease of the TIC pattern during MS acquisition.

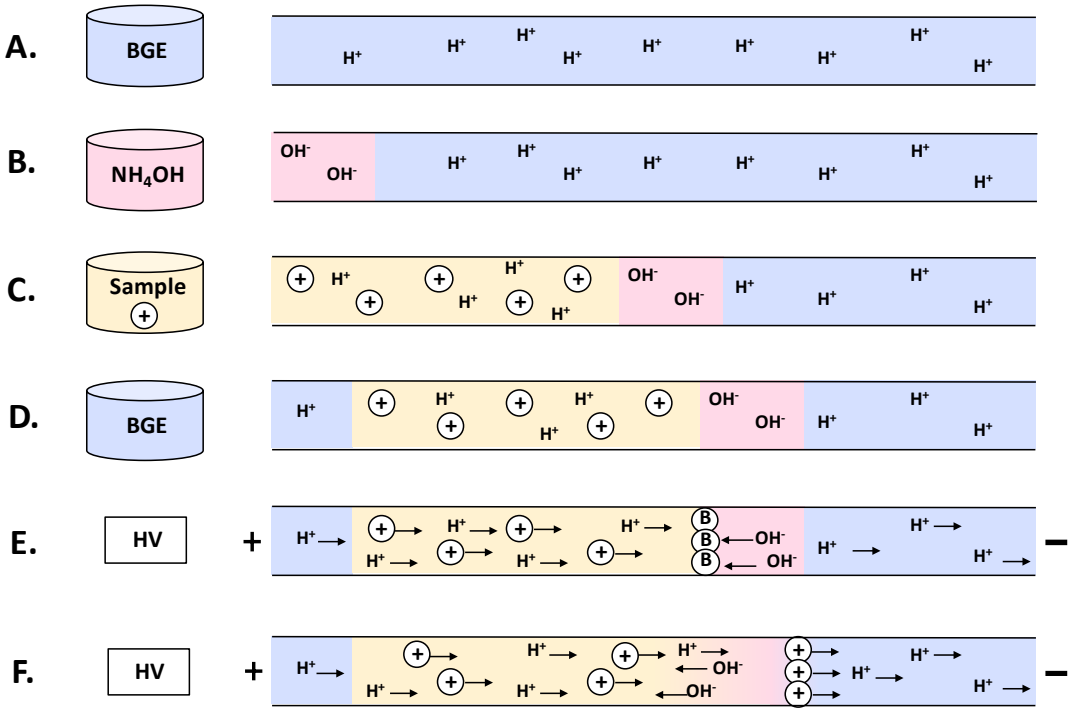

Figure 3.1. pH-mediated stacking. The capillary is first filled with BGE (A.). A small pre-plug of 7 % NH_4OH is injected, corresponding to 0.7 % of the capillary length (B.). A large plug of acidified urine sample containing the cationic compounds is injected, corresponding to 20.5 % of the capillary (C.). A post-plug of BGE is finally injected, corresponding to 0.2 % of the capillary length. All injections are hydrodynamically performed. When applying the separation voltage, the cationic analytes migrate through the sample until reaching the strong alkaline plug, become neutral, and stack in a narrow zone at the boundary of the sample (E.). The alkaline pre-plug is acidified by the sample and the acidic BGE, which allows for the beginning of the separation of the compounds returned at a cationic state (F.).

A favorable sensitivity enhancement (*ca.* 10 fold) was obtained compared to a conventional HD injection of diluted urine corresponding to 1-2 % of the capillary length. Moreover, the implementation of a pH-mediated stacking was well combined with a simple dilution of the sample, therefore avoiding a time-consuming and tedious sample preparation step. The stacking procedure was also found repeatable and was likewise used for the quantitation step where the potential variations were corrected by the use of deuterated IS.

2.2 Coated capillaries

2.2.1 Principle

Using low-pH BGEs, as already discussed in **Section 3.2.2.2** in the **Chapter I**, can lead to unstable analyses due to the absent or strongly reduced EOF [34,35]. The current instability can be prevented or even avoided with a careful selection of the sheath liquid composition. In this study, even using the same electrolyte in BGE and sheath liquid, the system stability was not ensured with the developed conditions. This instability was mainly observed with the CE-TOF/MS coupling where at least 50 % of the analyses presented a constant decrease of the CE current, probably due to the previously explained moving ion boundary effect. This was not the case with the CE-QqQ system used for the quantitation, where the CE current was fully stable over the time. Further investigations were carried out to determine the cause of this difference but without success. Differences in the geometry of the source (Dual ESI source *versus* Agilent Jet Stream Source) might possibly or partially explain these differences.

Not only the stability of the CE currents were insufficient but working at very low EOF also led to increased analysis time, which is deleterious for a screening or confirmation procedure, especially in clinical fields where a rapid identification is mandatory. Therefore, the use of coated capillaries was envisaged to enhance both stability and speed of analysis. Anionic capillary coatings provide the generation of a high and repeatable EOF towards the MS entrance, leading to an enhanced migration times' repeatability which is also advantageous for compounds identification. Furthermore, the coating can reduce the solute-surface interaction that can occur during the separation, especially with the injection of diluted matrix or the analysis of cations [36].

Numerous coating approaches can be implemented depending on the matrix, the compounds, and the analytical issues. Coating strategies will be deeply discussed in **Chapter IV** based on reducing protein adsorption but all the presented coatings can also be used for the analysis of low-molecular weight compounds. In this work, a bilayer anionic coating was selected for further investigations.

2.2.2 Anionic coating

Coated capillaries can be commercially purchased at a relatively high price or in-house generated by rinsing the fused-silica capillary with coating agents, often polymer solutions. Commercially available coated capillaries present the main advantage to be ready for use and do not need any recoating procedure after few runs. Depending on the polymer and coating composition, in-house coating processes involve:

(i) A coating procedure out of the CE instrument *via* rinses of the capillary with coating agents and rest periods (*e.g.,* cellulose-based coatings). The interactions involved between coating and capillary are covalent. This procedure is quite laborious but these coatings often do not require any re-coating after n runs and present a long-term stability;

(ii) A coating procedure within the CE instrument *via* rapid rinses of the capillary with polymer solution(s), which does not require any re-coating (*e.g.,* bilayer coating composed of polybrene, PB, dextran sulfate, DS, and/or poly(vinylsulfonate), PVS); or

(iii) A coating procedure within the CE instrument *via* rapid rinses of the capillary with polymer solution(s), which requires re-coating after n runs ($1 \leq n \leq 20$).

(ii) and (iii) are referred to as *adsorbed coatings*, while (i) includes *covalent coatings.* All these coatings procedures are categorized *static* as they are performed prior to the analysis and are not present in the BGE, compared to the *dynamic* coatings which involve the presence of the coating agent in the BGE during the separation [36,37].

A static-adsorbed and commercially available coating was selected for the screening step. The CEofix™ MS-compatible kit was purchased from Analis (Suarlee, Belgium) and is composed of two solutions, the *initiator* (INIT) containing polycations and the *accelerator* (ACCEL) composed of polyanions [38,39]. The exact composition of the INIT and ACCEL is unknown, although a PB-based INIT and a DS- or PVS-based ACCEL solutions are suspected. The procedure is depicted in **Fig. 3.2** and consists of a deprotonation of the silanols with a NaOH rinse followed by subsequent flushes with INIT and ACCEL, forming a bilayer anionic coating (*i.e.,* last layer of the coating being anionic).

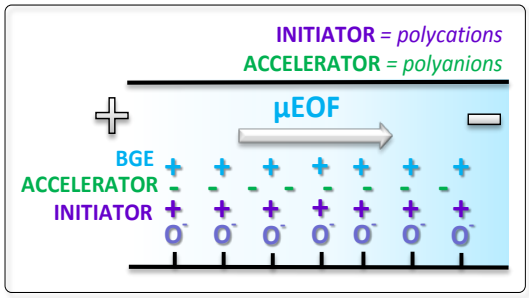

Figure 3.2. Schematic representation of the bilayer MS-compatible coating

The complete procedure was investigated to provide the best performance in terms of migration times' repeatability for the drugs of abuse. Recoating between runs and life time of the coating were also evaluated. The operating conditions are presented in **Table 3.1**.

Table 3.1. Coating procedure.

Coating procedure (ΔP = 2 bar)		
Methanol	5 min	(8.2 V_{cap})
Water	5 min	(8.2 V_{cap})
1M NaOH	5 min	(8.2 V_{cap})
Water	5 min	(8.2 V_{cap})
INIT	0.4 min	(0.7 V_{cap})
ACCEL	0.4 min	(0.7 V_{cap})
BGE	10 min	(16.4 V_{cap})
Recoating between runs (ΔP = 2 bar)		
Water	2 min	(3.3 V_{cap})
ACCEL	1 min	(1.6 V_{cap})
Overnight storing		
Capillary filled with BGE and tips placed in BGE vials		
Coating removal every 20 runs		
1M NaOH	5 min	(8.2 V_{cap})

After having discarded the first three analyses, the coating was stable over 20 runs. After 20 runs, the coated was easily and completely removed from the capillary with 1M NaOH before applying the whole coating procedure again. With this coating, the CE-MS was completely stable, the RSDs for migration times lower than 1 %, and the CE separation performed in less than 7 min. Taking into account the post- and pre-conditioning steps between runs, as well as the sample treatment and the injection, the whole CE-MS procedure was performed in less than 10 min. It is worth mentioning that this time does not include the application of the whole coating procedure every 20 runs (*ca.* 30 min). This coating is also attractive due to its commercial availability and the unnecessary bench preparation with ready-to-use solutions.

An arbitrary limit of 1 % for acceptable migration times RSDs was chosen to increase the reliability in compound identification. This is why the coating procedure was re-applied every 20 runs. Nevertheless, if a relatively higher RSD value is tolerated depending on the resolution and accuracy of the mass analyzer, a higher number of analyses can be performed with the same coated capillary with still acceptable repeatabilities (RSDs ≤ 3-5 %).

2.3 Sheath-flow interface

In parallel to the numerous developments and innovation proposed for the sheathless interface, the sheath-flow configuration has been currently seeing a new impulse for improvements. The first

sprayer commercially available for CE-MS coupling remains for many years unchanged. The shape of this triple-tube CE-MS sprayer was designed by modifying a conventional LC-MS nebulizer. Although adapted for stable and robust CE-MS hyphenation, this sprayer does not take into account the lower flow rates encountered in CE-MS (CE effluent and sheath liquid flow rate) than LC-MS. Therefore, a new adapted triple-tube sprayer was recently designed by Agilent Technologies aiming at enhancing the ionization efficiency and transmission. This sprayer was tested in this work prior to its commercialization by Agilent Technologies in 2012. Today, two commercial sprayers are thus available for the sheath-flow configuration.

The development of a new sprayer design was not only promoted by the need for sensitivity improvement, but also by the emergence of a new ESI source geometry based on the Jet Stream technology. The customers acquiring a recent MS instrument (such as the QqQ instrument used for the quantitation step) will automatically have this new source instead of the standard ESI source. However, the conventional triple-tube sprayer is not compatible with this new source. Thus, an evaluation of this new configuration (*i.e.*, new sprayer and new source) was essential to determine if any advantageous or deleterious effect on CE-MS hyphenation was observed. This evaluation is summarized in a Technical Note presented in **Appendix IV**.

2.3.1 Design of the triple-tube sprayer

Conventional triple-tube and new updated sprayer are schematized in **Fig. 3.3** with a zoomed picture of both sprayers' tips. Main modifications of the new sprayer are (i) the length of the needle and the sprayer, *ca.* 1 mm shorter, (ii) the design of the sprayer's tip, and (iii) the mechanical design, which helps position the needle exactly in the center of the sprayer's body.

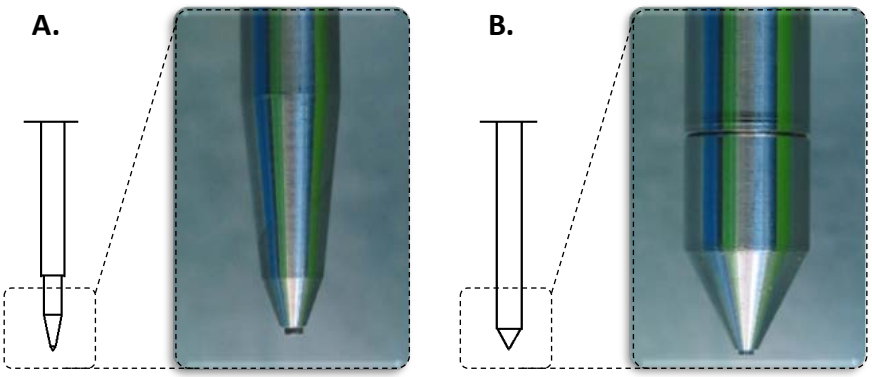

Figure 3.3. Schematic representations and zoomed pictures of the commercially available sprayers. A. Conventional triple-tube sprayer, B. Updated triple-tube sprayer.

These design modifications aimed at improving the efficiency of each step of the electrospray process, thereby enhancing the signal intensity. A systematic evaluation of the sprayer performed at various concentrations for model compounds showed significant differences in signal intensities, which were between 1.5- to 2-fold better for the new triple-tube sprayer. Both sprayers showed similar repeatability on peak area and heights, and the results were also confirmed with other copies of the at-this-time prototype sprayer which were available for the study. These results, although quite satisfactory, are mainly promising to encourage the development of even more adapted triple-tube sprayers to see further improvements in the ionization.

2.3.2 Electrospray ionization source geometry

Since the first ESI-MS experiments performed by Dole *et al.* in 1968, and besides the nanospray approach which will not be discussed here, numerous developments have been proposed to improve the ionization efficiency, including modifications of the source geometry and the sprayer position relative to the MS entrance, and addition of a heated gas to enhance the desolvation [40,41].

Today, three geometries are commercially available, depending on the position on the sprayer relative to the MS orifice, *i.e.*, (i) orthogonal geometry, where the spray is positioned at 90° relative to the x-axis between sampling capillary and first quadrupole (*e.g.*, standard or Jet Stream ESI sources from Agilent) (ii) off-axis geometry, where the spray is at 30-45° to x-axis (*e.g.*, Ion Max source from ThermoFisher, Turbo Ion Spray from AB Sciex), and (iii) Z-spray geometry, presenting a double orthogonal sampling (*e.g.*, Zspray from Waters). Orthogonal and Z-spray configurations provide better performance than off-axis sources due to the prevention of clogging of the MS orifice by non-volatile materials, as well as an increased sensitivity [40-42].

The Agilent sheath-flow interface for CE-MS hyphenation is based on the orthogonal configuration and is the only configuration commercially available. In 2010, Klampfl and co-workers investigated the influence of the sheath-flow axis in CE-ESI-TOF/MS with in-house modified interfaces compared to the orthogonal co-axial sheath-flow interface from Agilent [43]. The two in-house configuration are illustrated in **Fig. 3.4** and were composed of (i) an in-axis 0° (**Fig. 3.4.A**) and (ii) an in-axis 45° sprays (**Fig. 3.4.B**), which were coupled to a Mariner TOF/MS system from PerSeptive Biosystems. The same conventional ESI triple-tube sprayer was used in each configuration. Due to the design of the MS instrument with an application of the ESI high-voltage to the sprayer needle (and not to the MS orifice, as for Agilent or Bruker instruments), a way of grounding the CE current had to be found for these in-house configurations, which was done by using an appropriate resistor (> 70 MΩ). With the same sheath-liquid composition and flow rate (2 µL/min) for each set-up, LODs obtained for the analysis of fluoroquinolones were up to 20-fold better with the in-axis configurations, probably explained by the shorter distance between spray tip and MS orifice.

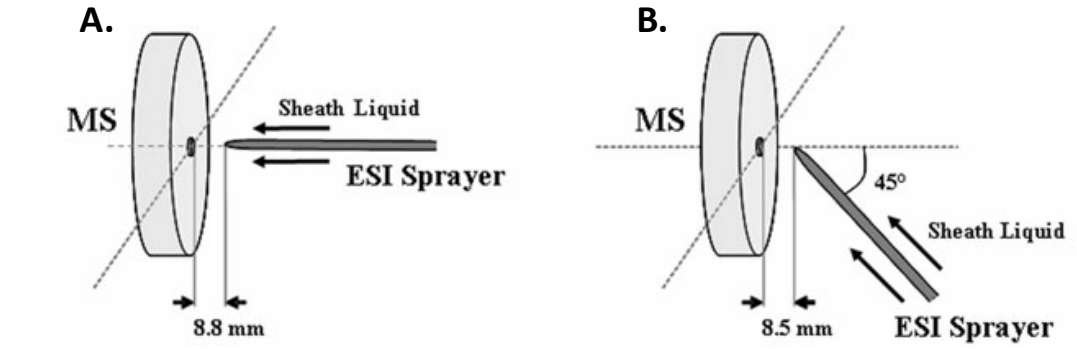

Figure 3.4. View from the top of the in-house configuration with their respective distance to MS orifice and angle between MS-inlet axis and sprayer. A. In-axis sheath-flow interface (0°), B. In-axis sheath flow interface (45°). Reprinted from [43].

The addition of a nitrogen heated gas has also been considered to increase the desolvation efficiency and was thus widely employed by manufacturers in response the emergence of fast LC-MS (higher flow rates). The Turbo V™ Ion Source from AB Sciex contains two auxiliary heated nitrogen sources

(referred to as *drying gas*) placed at 45° to each side of the sprayer. The Ion Max source from ThermoFisher contains both so-called *sheath gas* and *auxiliary gas* which flow through the probe. The sheath gas is an inner coaxial nitrogen "conventional" nebulizing gas, while the auxiliary gas is an outer coaxial nitrogen sheath gas that helps the former in droplets desolvation. This mode of operation has been referred to as *Heated-Electrospray Ionization* by ThermoFisher [40,42]. Agilent Technologies launched few years ago a new ESI source based on the Jet Stream thermal gradient focusing technology. In this source, a superheated nitrogen sheath is added to enhance the desolvation efficiency while confining the nebulizer spray, as illustrated in **Fig. 3.5**. The ion efficiency is increased by two mechanisms, (i) the thermal gradient aerosol focusing, and (ii) the modification of the nebulizer current by additional nozzle voltage. Both improvements have shown a 5- to 10-fold sensitivity enhancement in LC-MS with LC flow rates between 0.25 to 2 mL/min.

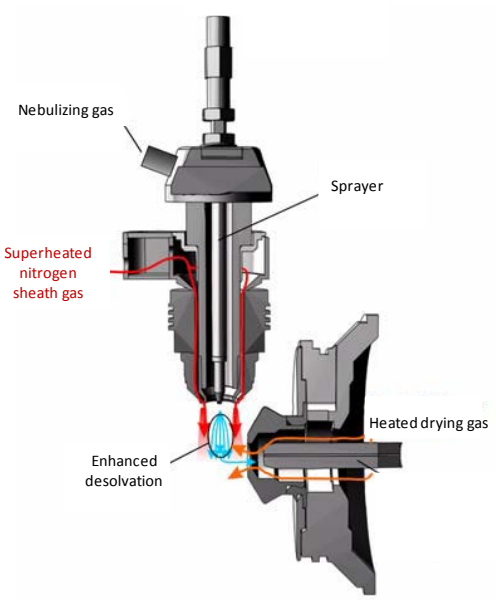

Figure 3.5. Agilent Jet Stream technology. Reprinted from [44].

The thermal gradient focusing is represented by the heat transfer to the border between the condensed phase plume and the heated sheath gas. The radial dimension R of the generated aerosol is inversely proportional to the temperature difference ΔT between the sheath gas and the boiling temperature of the liquid within the plume according to **Eq. 3.4** [45]:

$$R \sim \frac{1}{\sqrt{\Delta T}} \qquad \text{(Eq. 3.4)}$$

Thus, the more the temperature of the sheath gas is increased (generally 350 °C with a flow rate of 11 L/min); the more the spray condensed phase plume is focused. When the highly charged aerosol of droplets and ions is confined, the ion density in front of the MS entrance is increased.

The second improvement is the presence of a conductive sheath nozzle around the nebulizer. Applying a voltage on the nozzle ($0 \leq V \leq 2000$) can significantly change the electrical field close to the nebulizer's tip, resulting in changes in the total nebulizer current and total charge for each droplet. The net charge in the aerosol is not only responsible for the ions production but also for the

Coulombic repulsion. In conventional ESI source, the nebulizer current is not well controlled and can vary according to the effluent and the potential difference between the needle and the MS entrance [45].

An interesting work was proposed by Dugourd and co-workers who investigated the pH changes in the electrospray plume observed in AJS source by optical spectroscopy [46]. A laser-induced-fluorescence profiling set-up was implemented on a modified AJS source which allowed to profiling the pH state of the droplets as they evaporated in the electrospray plume by measuring the emission spectra of a pH-sensitive fluorescent dye (C.SNARF-1). A pH-chromic dye presents different fluorescence spectra between acid and basic forms. First, the implemented system was tested by measuring the fluorescence emitted by Rhodamin 6G, a non-chromic dye. The results obtained with an effluent flow rate of 50 µL/min, and sheath gas temperature and flow rate fixed at 180 °C and 7 L/min, respectively, are illustrated in **Fig. 3.6.A**. Depending on the sheath gas parameters, the ESI profile changed in its dimension with an increased confinement when enhancing the gas flow rate, showing a better evaporation of the droplets.

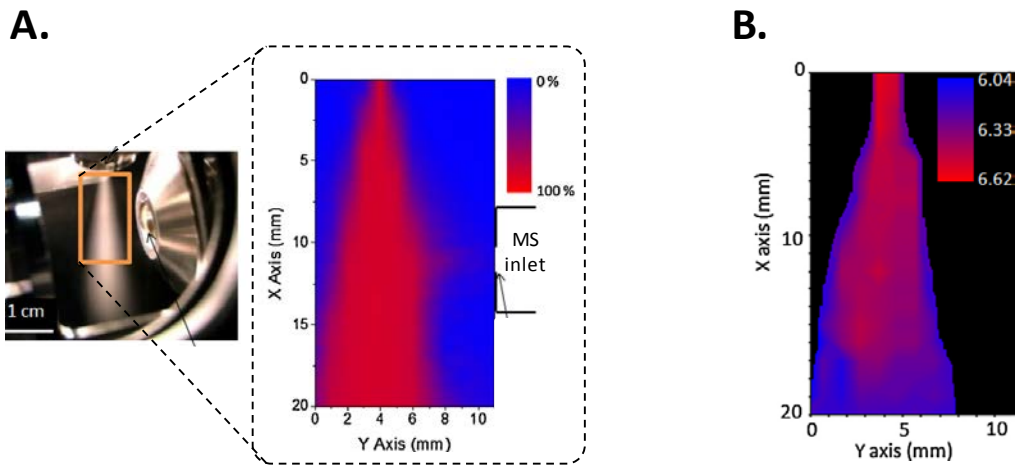

Figure 3.6. Jet Stream evaluation. A. Picture of the ESI plume and measurement of the fluorescence emitted by Rhodamin 6G in methanol/water (80:20, *v/v*) infused at 50 µL/min. The sheath gas flow rate and temperature are fixed at 7 L/min and 180 °C, respectively. B. $\boldsymbol{XY}$image of the pH in the plume by measuring the emission spectra of 250 µM C.SNARF-1 in water at an initial pH of 6.5, and same sheath gas parameters than in A. Adapted from [46].

Then, optical measurements of the pH-sensitive dye, C.SNARF-1, clearly indicated that the pH within the droplets was modified as they moved down the plume, as shown in **Fig. 3.6.B**. Moreover, a lateral heterogeneity was observed with a lower pH at the edge of the spray plume than in the center. Modification of the pH was explained by the solvent evaporation in the droplets. The presence of the heated sheath gas enhanced this phenomenon, and, thus, the pH modification. The authors found that improving the desolvation process of solutions having an initial pH > 7 induced an increase in droplets pH while solution with pH < 7 resulted in decreased droplets pH; the higher the sheath gas flow rate and temperature, the more important the effect in pH modification was observed.

This phenomenon is probably also observed when using AJS source for CE-MS and might be more significant due to the higher differences in flow rates between CE effluent and sheath gas. The AJS source was compared to the conventional ESI source in a systematic study with the analysis of model

compounds, summarized in **Appendix IV**. Best conditions for signal intensities were obtained with the sheath gas flow rate and temperature set at 3.5 L/min and 195 °C, respectively. With ESI negative ionization mode, performance of the AJS source was found better with up to 4-fold improvement in sensitivity. With ESI positive ionization mode, the results were either comparable or slightly better for the standard ESI source, depending on the compounds. When correcting the signals with deuterated IS, no significant difference in signal intensities between both sources was observed. As the AJS source was used with CE-QqQ for the quantitative step, the systematic IS correction was in any case applied, and the coupling enough sensitive for the selected application. The IS correction was also required in the developed quantitative procedure due the ion suppression measured for MTD according to Matuszewski's methodology, with a matrix effect of 73 ± 5 %. In this study, only the matrix effects observed with AJS source were evaluated during the method validation. In a recent study, the matrix effects observed with both standard ESI and new AJS sources were qualitatively compared [41]. As illustrated in **Fig. 3.7**, post-column infusion experiments showed stronger ion suppression with AJS source over the whole chromatogram, and mainly in the region of typically low matrix effects. Therefore, the presence of the sheath gas seemed to enhance the signal intensities of targeted compounds, which were confirmed by the authors, but also increase the ionization of the matrix components.

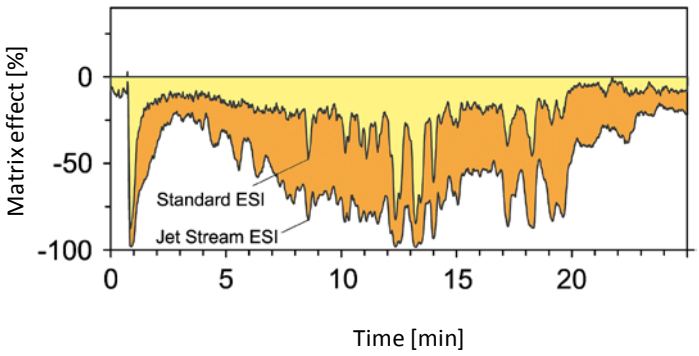

Figure 3.7. Comparison of the matrix effect profiles observed for standard ESI and AJS source. The sample consisted of 20 mg of orange extracted by methanol followed by a clean-up on diatomaceous earth with dichloromethane. Reprinted from [41].

In conclusion, the Jet Stream technology has shown to provide some sensitivity improvement (probably depending on the matrix) in LC-MS. As this source is not dedicated for CE-MS and was not designed for this purpose, some modifications are still required to see a similar improvement in the performance, mainly the possibility of applying lower values of sheath gas temperature and flow rate. Indeed, recommended values for sheath gas flow rate and temperature in LC-MS are 11 L/min and 350°C, respectively, whereas best results in signal intensities were obtained in CE-MS with values less than 3.5 L/min and 200°C, respectively.

2.4 Mass analyzers

2.4.1 Time-of-flight mass spectrometry

As discussed in **Chapter I**, in STA procedures, the choice of MS detection is oriented towards mass analyzers providing a high mass resolution and a high mass accuracy, as encountered with TOF

analyzers. The quite simple principle behind TOF/MS technology was proposed in 1946 by Stephens and the first instrument developed by Cameron and Eggers in 1948. A TOF analyzer relies on the separation of ions moving in the same direction in a field-free region (the so-called *flight tube*) and presenting different velocities. The principle is explained here for a TOF/MS coupled with an ESI source (continuous ion source). Under the influence of an external electric field created by using parallel plates (one being an extraction grid to let the ions pass through) at different potentials, a discrete packet of stationary ions (temporal distribution) is accelerated to a constant kinetic energy and moves towards the detector at a velocity inversely proportional to the square root of the *m/z* ratio [47,48]. The kinetic energy E_c acquired by an ion presenting a mass m and a total charge $q = ze$ (where z is the number of electron charge and e the charge of electron, *i.e.*, -1.60×10^{-19} C) is equal to its potential energy E_p observed after the application of the acceleration potential V_s according to **Eq. 3.5**:

$$E_c = \frac{1}{2}mv^2 = E_p = qV_s = zeV_s$$

For a given E_c, smaller masses will thus have larger velocities v, and arrive at the detector earlier than large masses. The time t required by an ion to reach the detector is defined by **Eq. 3.6**:

$$t = \frac{d}{v} \qquad \text{(Eq. 3.6)}$$

Where d is the flight distance. Combining **Eq. 3.5** and **Eq. 3.6** leads to the **Eq. 3.7** which expresses the mathematical relation between t and m:

$$t^2 = \frac{m}{z}\left(\frac{d^2}{2eV_s}\right) \qquad \text{(Eq. 3.7)}$$

With $d^2/2eV_s$ = constant.

Therefore, the linear TOF mass analyzer provides a theoretically unlimited mass range and a rapid analysis of a wide *m/z* range with a good sensitivity due to its dispersive properties (compared to a scanning approach). However, the linear configuration suffers from a poor resolution (< 2000 FWHM), due to non-ideal configuration parameters (such as acceleration fields) affecting the dispersion of the time-of-flight, including temporal distribution, spatial distribution, and energy (or kinetic) distribution. The temporal distribution is observed for ions presenting the same *m/z*, the same v, and accelerated from the same position but entering the tube at different time. The spatial distribution occurs with a variation of the initial position of ions with same *m/z* in the extraction field when first accelerated. Finally, a variation in initial ions velocity under the influence of an external electric field leads to a difference in the duration of acceleration, producing a difference in their energy distribution [47,49,50]. The technological developments in the 1980s-1990s including reflectron, delayed extraction, and orthogonal acceleration allowed to significantly improve the resolution R of TOF analyzers, given by **Eq. 3.8**:

$$R = \frac{m}{\Delta m} = \frac{t}{\Delta t} \approx \frac{d}{2\Delta z} \qquad \text{(Eq. 3.8)}$$

Reflectron technology was first proposed by Mamyrin who patented an ion mirror device at the end of the tube to focus the energy and improve the resolution. The reflectron is constituted by a long series of ring electrodes that create a homogenous decelerating electric field (opposed to the accelerating field) to reverse the direction of the ions travel. For ions of the same *m/z* entering the reflectron, ions with higher kinetic energy will spend more time in the electric field (further penetrating the reflectron) than ions with lower energy, and then re-emerge with a reversed velocity. The angle of the ion entry into the mirror is adjusted slightly away from 90° to ensure that the ions follow different paths after being reflected, thus allowing for positioning the detector on a different axis than the ion source [47]. Reflectrons provide also a higher resolution by increasing the flight distance d according to **Eq. 3.8** without modifying the size of the instrument, but at the expense of the sensitivity and the mass range.

The principle of delayed extraction was proposed to reduce the dispersion in the time-of-flight of ions sharing the same *m/z* and consists of introducing a time delay between the time of formation of the ion in the source and their extraction. In a constant extraction, the ions are instantaneously accelerated after being formed. This is not the case in the delayed exaction where ions are formed in the free-field zone and are allowed to disperse during hundreds of ns before the onset of the acceleration electric field. An ion *a* which is further than an ion *b* from the extraction grid starts to be accelerated at a higher potential, meaning that *b* exits earlier the acceleration zone. When *a* exits the acceleration region, it will be accelerated at a greater velocity than *b*. Thus, delayed extraction allows the ion *b*, which was closer to the extraction plate and left the ion source earlier but with a lower velocity, to reach the detector at the same time than ion *a* which left the source later but has a greater velocity. The ion source parameters have to be properly adjusted to allow the detector to detect their simultaneous arrival. The delayed extraction improves the resolution and the mass accuracy without having a negative effect on the sensitivity. However, issues in mass calibration can be encountered due to this delay, and it is not really efficient for higher masses [50].

Coupling continuous ionization sources such as ESI with intrinsically pulsed processes as encountered with TOF/MS is rather complicated, and was rendered more readily possible with the application by Guilhaus, Dawson, and Dodonov of orthogonal acceleration (oa) in the 1990s [51]. In oa-TOF/MS, ions are orthogonally injected in the flight tube with an orthogonal accelerator. Ions are first focused into a nearly parallel beam in the ion source and transferred to an orthogonal accelerator, which accumulates the discontinuous packets of ions. A voltage is then applied, inducing a strictly orthogonal acceleration field, which promotes the flight of the ions in the direction of the flight tube and reducing the distribution in the initial velocities. During the time-of-flight of this ions packet, the orthogonal accelerator is filled with other ions, which are accelerated when the first packet arrives at the detector. Oa-TOF/MS thus provides a high efficiency in gating ions from a continuous source with a simultaneous correction of spatial and velocity dispersion, with resolving power between 10,000 and 20,000 FWHM [49].

Fig. 3.8 depicts the Agilent 6210 LC/MSD TOF which was used during this work for the screening step and benefits from the previously described technical developments [52].

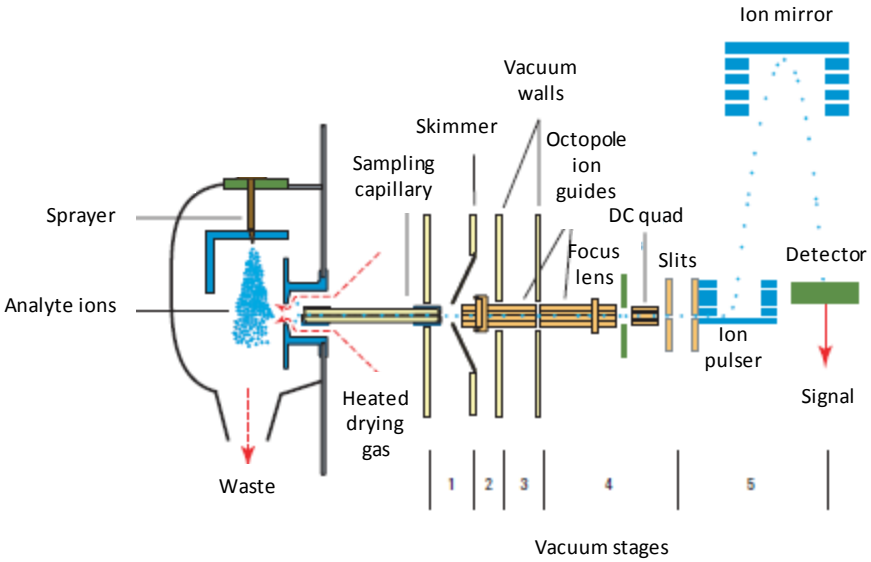

Figure 3.8. Schematic representation of Agilent 6210 LC/MSD TOF System. Reprinted from [52].

Ions produced in the ESI source are electrostatically drawn through the heated sampling capillary into the first stage of the vacuum system. A metal skimmer with a small hole allows for the heavier ions with great momentum passing through the aperture while the lighter nitrogen gas molecules are pumped away. The ions enter in the second stage of the vacuum system and are immeditaly focused by the first of the two octopole ion guides *via* application of RF voltages to the metal rods of the octopole, confining the ions to the open center of the rods assembly. As the ions pass through the first octopole, they enter in the third stage of vacuum. In the fourth vacuum stage, they immediately enter the second octopole ion guide, which accelerates the ions *via* DC potential. The ions then arrive to the beam-shaping optics, where they are focused with a focus lens and a DC quadrupole to achieve optimal parallelism and size. The nearly parallel beam enters then in the last vacuum stage, and passes through a pair of slits to reach the ion beam pulser, composed of a stack of plates, each having a hole except the back plate. The high-voltage pulse is applied to the back plates, accelerating the ions through the stack of pusler plates towards the flight tube, equipped with a reflectron. Ions finally reach the detector, whose first stage is composed of a microchannel plate (*i.e.*, a thin plate perforated by microtubes). Each microchannel acts as an electron multiplier (*ca.* 10 e^- for every incoming ion). Electrons are accelerated onto a scintillator that emits photons when struck by the electrons. The photons are focused through optical lenses onto a photomultipler tube which amplify the number of photons, and produces an electrical signal proportionnal to the number of photons. The conversion from electrical to optical and back again electrical signal is used to isolate the flight tube and the detector [52].

For each time that a high voltage is applied to the back plate of the ion beam pulser, a single spectrum called *transient* is recorded. Each transient is added to the previous one until a defined number has been obtained, depending on the mass range and the data acquisition rate. With rate of 1 spectrum/s and a standard mass range (100-3200 *m/z*), 10,000 transients are recorded to give a spectrum. Increasing the data acquisition rate leads to a smaller number of transients. The more the mass range is extended, the longer it is necessary to wait until the last mass arrives at the detector before triggering the ion pulser again, thereby decreasing the number of transients.

The conversion of the ion pulses detection into a digital measurement is performed with an analog-to-digital converter (ADC) system. With ADC systems, the signal from the detector is digitized at a fixed sampling rate, *i.e.*, 1 GHz (one record every 1 ns). The digital value recorded is represented by 8-bit value, corresponding to a dynamic range of 2^8 counts (0 to 255 counts). ADC system stores each conversion in memory, and each time the pulser fires, it adds the new measurement to the ones already recorded from the previous transients. ADC system thus acts as an integrating transient recorder. With ADC conversion, the detector output signal is accurately recorded, whether the signal comes from a large or a small ion current [47,52]. Therefore, detectors based on the ADC conversion lead to an extended dynamic range. The parameters have to be carefully adjusted because even in absence of ions, a signal can be measured (system noise). This can be done by biasing the detector amplifier to a value close to zero so that when no ion is present, no data is measured. With ADC recorder, three-order linear dynamic ranges can be obtained with acceptable mass accuracy over the whole range. This is often still insufficient for quantitation purposes, which *inter alia* explains the limited use of TOF/MS in quantitative procedures [48].

In order to ensure the mostly accurate measurement of *m/z* ratios, a mass calibration procedure is performed every day. The calibration procedure consists of measuring the time-of-flight of different ions (between 5 and 10) of known exact masses, defining a daily calibration curve which allow for the calculation of exact masses related to time determinations. After the calibration procedure, the remaining error is usually less than 1 ppm over the range of calibration. Even with a sufficient mass calibration, instrumental drifts factors can occur, leading to noticeable shifts. They can be corrected with the use of reference mass recalibration during the analysis. In the 6210 LC/MSD TOF system, this can be done by introducing two known masses at a given concentration into the ion source *via* a second nebulizer and an automatic recalibration of the system is continuously performed. The known masses can be also directly added to the sheath liquid in case of CE-MS analysis, without the need for a Dual source configuration. The mass accuracy is also related to the resolution; in case of low mass resolution, a peak of interest can be merged within the chemical background, and a small unresolved impurity can shift the centroid data of the expected mass, leading to inaccurate measurement [52].

2.4.2 Triple quadrupole

As for STA procedure, the selection of the suitable mass analyzer for compounds quantitation relies on numerous parameters, including sensitivity, selectivity, scan speed, mass resolution, and instruments costs [53]. In routine laboratories, QqQ in SRM mode remains widely used, providing outstanding quantitation performance with very rapid duty cycles (10-50 ms), allowing for a sufficient number of data point for each analyte and an acceptable resolution, sensitivity, selectivity, and high dynamic range [54]. Performance in resolution and sensitivity is not comparable with the recent developments in HRMS instruments, but they are adequate for quantitation purposes and QqQ instruments remain much less expensive than HRMS instruments [55].

In this work, the Agilent 6490 Triple Quadruple LC/MS system was used, which comprises the most recent innovations proposed by Agilent Technologies to increase the system performance, *i.e.*, the AJS source, a hexabore sampling capillary, and the high pressure/low pressure ion funnels, and is depicted in **Fig. 3.9**.

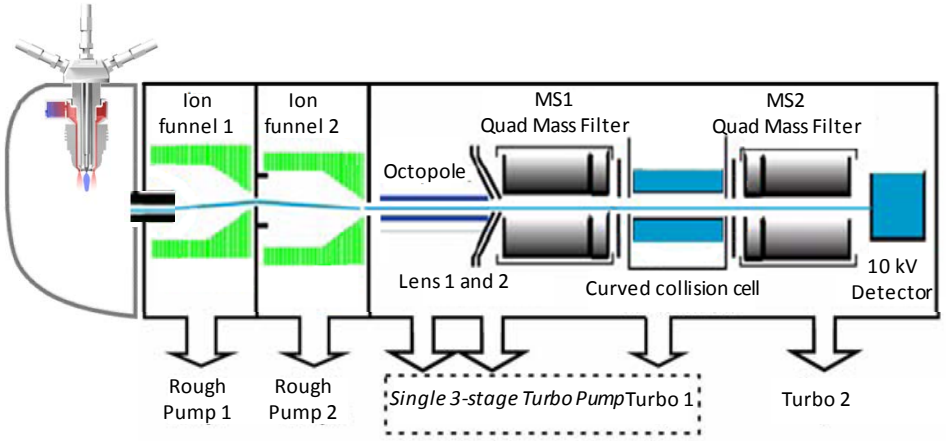

Figure 3.9. Schematic representation of Agilent 6490 Triple Quadrupole System with AJS source, hexabore capillary, and ion funnels. Adapted from [56].

On conventional MS instruments, the ions produced in the ESI source are sampled by a single inlet restricting capillary of *ca.* 600 µm i.d. The hexabore sampling capillary is a shorter assembly (*ca.* 400 µm) composed of six independent capillaries that are used to enhance the ion sampling efficiency. Not only are more ions sampled, but also the majority of the gas from the source region towards the ion optic system. In order to remove the excess of sampled gas and to further increase the ion transmission efficiency, a dual-stage ion funnel system has been added after the hexabore assembly, replacing the conventional skimmer. The first ion funnel has a pressure between 7 and 14 torr, while the second is a low pressure funnel at 1-3 torr. The skimmer typically used in conventional mass analyzers to separate the first and the second vacuum chambers samples only a limited fraction of the ion cloud and thus provides a limited ion transmission [2]. With ion funnels, the ion transmission is increased by an efficient capture of ions entering the first vacuum stage. An ion funnel is composed of a series of closely spaced cylindrical ring electrodes whose i.d. gradually decrease, allowing for a confinement of the ions as they pass through the funnel. The progressively smaller i.d. enable the efficient focusing of the spatially dispersed ion cloud to a much smaller radial size [1,2,57]. Ions are guided by application of RF potentials of opposite polarity to adjacent electrodes and DC potential gradients to the ring electrodes in order to drive the ions along the axis.

Passing through the RF octopole ion guide and the first quadrupole Q1, the ions reach the collision cell to be fragmented. This hexapole curved collision cell includes a tapered cell structure which increases the ion acceptance while reducing the generated noise. A second turbopump (*Turbo 2*, see **Fig. 3.9**) is added to the last vacuum stage to help pump out the excess of gas load coming from the curved collision cell. Compared to older instruments, the third quadrupole Q3 presents improved drive electronics. More ion motion cycles can be produced at a higher drive frequency which leads to a better resolution [56]. However, this higher drive frequency reduces the mass range of the 6490 Triple Quadrupole below 1400 *m/z*. This limited mass range does not present an issue for low-molecular weight compounds analysis but is clearly disadvantageous for protein determination. Finally, the detector is fixed orthogonally to the ion beam coming from Q3, which reduces the possibility of impact with neutral molecules. The detector is composed of two dynodes which convert the ions to electrons prior to impacting the multiplier [56].

With these technological improvements, the QqQ provides a maximum acquisition speed of 10,000 amu/s and an expected dynamic range up to six orders of magnitude [42]. It also presents an interesting feature referred to as *Triggered Dynamic MRM*, where confirmatory (secondary) SRM transitions can be automatically triggered and acquired when the abundance of a primary SRM transition is higher than a fixed threshold, similar to the data-dependant acquisition described in **Chapter I**.

2.5 Introducing Article III

Article III presents the complete methodology involved in the development of a multi-target screening approach by CE-ESI-TOF/MS for the determination of drugs of abuse and their relevant metabolites in urine, followed by the confirmation and quantitation within the same step by CE-ESI-MS/MS. The article describes the results obtained for each method improvements described in **Section 2**.

The screening step involved a simple urine dilution with BGE and water to normalize urine pH and lower the urinary salts content prior to CE injection. The BGE consisted in 1M formic acid at pH 1.8, ensuring the maximal ionization of the screened compounds. With an optimized on-line preconcentration technique, a pH-mediated sample stacking, more than 20 % of the capillary length was filled during the HD injection (*ca.* 320 nL of sample). For the latter, numerous injection parameters were investigated, such as NH_4OH concentration, and plug lengths. Using a coated capillary allowed for an enhanced migration's time repeatability, with RSDs lower than 1 % for the screened compounds. Moreover, due to the generation of a high EOF, all compounds were separated in less than 7 min. Therefore, the overall analysis, including the sample pre-treatment, the separation, and the detection, was performed in less than 10 min per sample. With the developed conditions, estimated LODs were as low as 2 ng/mL, ranging for example from 2 to 50 ng/mL for amphetamines, and from 10 to 50 ng/mL for COC and its respective metabolites.

The quantitation step, which also served as confirmation of the presumed positive samples of the screening procedure, was performed by CE-MS/MS with a QqQ which was equipped with both new sprayer and AJS source. Sample dilution, BGE composition, and injection parameters were the same as the screening step, while fused-silica capillaries were used to increase the selectivity of the separation regarding co-migrating isobaric compounds. COC and MTD, as well as their respective deuterated IS, were selected as model compounds to illustrate and validate the quantitative procedure. The most sensitive SRM transition for each compound was selected for the quantitation. It has to be noticed that for confirmation purpose, a higher number of transitions should be required, but an increase in the collision energy during CID led to a high number of fragments presenting very poor sensitivity. Thus, for this procedure, only the most sensitive transition was selected to ensure a reliable and accurate quantitation. The procedure was evaluated according to FDA guidelines based on selectivity, response function, trueness, precision, and accuracy. Selectivity involved the evaluation of matrix effects which were quantified according to Matuszewski's quantitative procedure [58]. COC did not show any significant matrix effect, however, with a signal suppression of 27 ± 5 %, MTD was prone to a deleterious effect of the co-migrating interferents. Therefore, the systematic use of IS correction as well as external calibration within the reconstituted matrix was selected for the quantitation.

Detailed results obtained for the validation process can be found in **Article III**. As a summary, **Fig. 3.10** presents the relative accuracy profiles obtained for both compounds, expressing the total error of the method with an accepted risk $\propto = 5\,\%$ and acceptance limits $\lambda = \pm 30\,\%$ for each level of concentration.

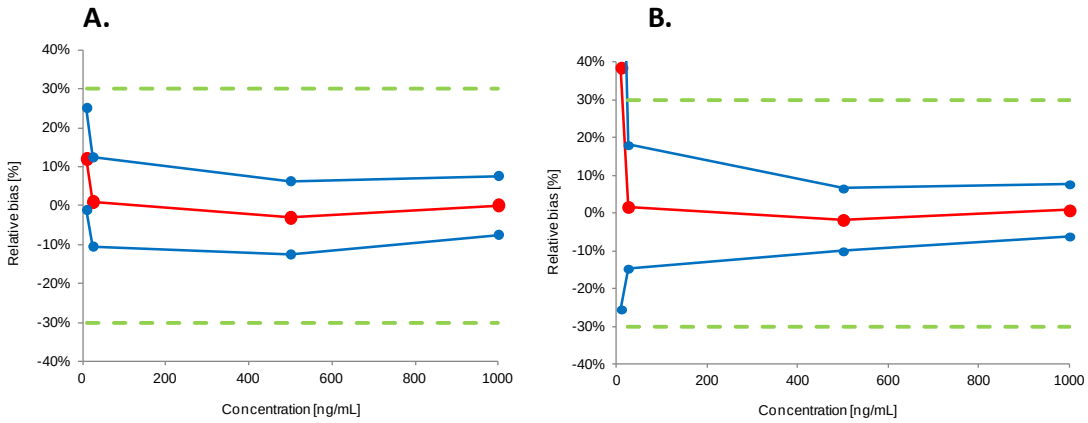

Figure 3.10. Relative accuracy profiles obtained for A. COC and B. MTD. Red line expresses the trueness, blue lines the lower and upper confidence limits of the mean bias, and green dashed lines the ±30% acceptance limit.

As illustrated in **Fig. 3.10.A**, the lower and upper confidence limits of the mean bias for COC were included within the acceptance limits for each level of concentration; the method was thus accurate for the quantitation of COC between 10 and 1000 ng/mL. For MTD, shown in **Fig. 3.10.B**, the lower concentration was not included within the acceptance limits and the LLOQ was thus interpolated from the absolute accuracy profile and defined at 21 ng/mL. Therefore, MTD quantitation was accurate between 21 and 1000 ng/mL. The validated ranges were fully applicable to clinical or forensic field, as demonstrated with the quantitation of previously screened samples containing COC and MTD. It is worth mentioning that COC and MTD are not systematically determined during typical forensic or clinical procedures which are more focused on the determination of their respective and specific metabolites (*e.g.*, benzoylecgonine, norCOC, or EDDP). Nevertheless, COC and MTD can be both detected in urine after their respective consumption and their quantitation thus remains useful [59,60].

The complete quantitative procedure was also published in an Application Note which is presented in **Appendix V**.

3 Conclusions

An exhaustive CE-MS strategy was proposed for the multi-target screening of drugs of abuse in urine followed by their quantitation, illustrated here for COC and MTD. The CE-ESI-TOF/MS and CE-MS/MS developed methods showed attractive performance, allowing for a high sensitive determination with rather low LODs and LLOQs. Both methods were presented in **Article III** as a two-step methodology consisting in a first multi-target screening step followed by confirmation/quantitation of presumed positive samples. The proposed CE-ESI-TOF/MS method may be obviously used as a confirmation step following an immunoassay determination, whereas the CE-MS/MS procedure can be applied for

quantification purpose after LC-MS or GC-MS screening. Two model compounds were used for the validation process but other drugs of abuse or their metabolites could also be quantified after proper validation procedure. Urine matrix was used for both screening and quantitative step. As stated in **Section 2.4.1.1** of **Chapter I**, urine is not frequently considered for quantitation compared to serum or plasma. With some adjustments in the sample pre-treatment procedure and the injection, the developed CE-MS/MS method may also be applied to serum, plasma, or even oral fluid specimens.

Nearly all the technical or method improvements proposed in **Section 2** were found to significantly enhance the overall performance, whether by enhancing the sensitivity or the analysis throughput. Only the AJS source was considered deleterious with lower ionization intensities obtained *versus* the conventional ESI source in positive ionization mode. If the sensitivity is a crucial parameter, it is thus recommended to perform the experiments with the new triple-tube sprayer but the conventional ESI source. Regarding the AJS source, some improvements are presently required by the manufacturer to provide not only an enhanced sensitivity in LC-MS but also in CE-MS configuration.

Finally, it has to be noticed that the proposed screening and quantitation procedure are fully applicable to the analysis of basic compounds (pKa ≥ 2-3) but are not usable for the analysis of acidic or neutral compounds, such as LSD, or very weak bases, such as a large number of BZD. This is why the screening procedure is referred in this Chapter to as a *multi-target screening* approach, and not a *general unknown screening*. For BZD or acidic compounds analysis, CE-MS may still be envisaged provided that modifications of the BGE composition and ton-line sample preconcentration are carried out, as well as MS parameters.

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5 Scientific publication

Article III

Highly sensitive capillary electrophoresis-mass spectrometry for rapid screening and accurate quantitation of drugs of abuse in urine

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Highly sensitive capillary electrophoresis-mass spectrometry for rapid screening and accurate quantitation of drugs of abuse in urine

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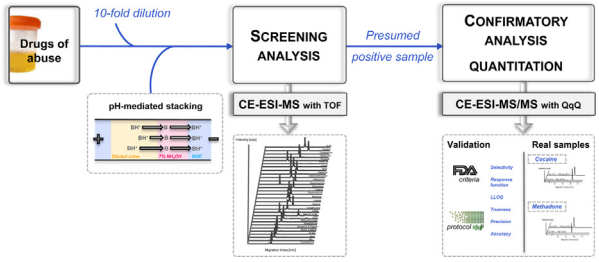
HIGHLIGHTS

- A CE-MS two-step analytical strategy was implemented in bioanalysis.
- CE-ESI-TOF/MS was implemented for the screening step with on-line pre-concentration.
- CE-ESI-MS/MS with QqQ was used for quantitation and was validated for COC and MTD.
- QqQ was equipped with a new sprayer and a new ESI source.
- The two-step workflow was applied to an analysis of toxicological cases.

GRAPHICAL ABSTRACT



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ABSTRACT

The combination of capillary electrophoresis (CE) and mass spectrometry (MS) is particularly well adapted to bioanalysis due to its high separation efficiency, selectivity, and sensitivity; its short analytical time; and its low solvent and sample consumption. For clinical and forensic toxicology, a two-step analysis is usually performed: first, a screening step for compound identification, and second, confirmation and/or accurate quantitation in cases of presumed positive results. In this study, a fast and sensitive CE-MS workflow was developed for the screening and quantitation of drugs of abuse in urine samples. A CE with a time-of-flight MS (CE-TOF/MS) screening method was developed using a simple urine dilution and on-line sample preconcentration with pH-mediated stacking. The sample stacking allowed for a high loading capacity (20.5% of the capillary length), leading to limits of detection as low as 2 ng mL^{-1} for drugs of abuse. Compound quantitation of positive samples was performed by CE-MS/MS with a triple quadrupole MS equipped with an adapted triple-tube sprayer and an electrospray ionization (ESI) source. The CE-ESI-MS/MS method was validated for two model compounds, cocaine (COC) and methadone (MTD), according to the Guidance of the Food and Drug Administration. The quantitative performance was evaluated for selectivity, response function, the lower limit of quantitation, trueness, precision, and accuracy. COC and MTD detection in urine samples was determined to be accurate over the range of 10–1000 ng mL^{-1} and 21–1000 ng mL^{-1}, respectively.

Abbreviations: AEME, anhydroecgonine methyl ester; AJS, Agilent Jet Stream; BE, benzoylecgonine; BGE, background electrolyte; CE, capillary electrophoresis; COC, cocaine; CS, calibration standard; D-PX, d-propoxyphene; EDDP, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolydine; EIE, extracted ion electropherogram; EOF, electroosmotic flow; GC, gas chromatography; HD, hydrodynamic; *i*-PrOH, isopropanol; IS, internal standard; LC, liquid chromatography; LLE, liquid–liquid extraction; LLOQ, lower limit of quantitation; *m/z*, mass-to-charge ratio; MA, methamphetamine; 6-MAM, 6-monoacetylmorphine; MBDB, N-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine; MDA, 3,4-methylenedioxyamphetamine; MDEA, 3,4-methylenedioxyethylamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; ME, matrix effect; MeOH, methanol; MS, mass spectrometry; MTD, methadone; OLS, ordinary least square; QqQ, triple quadrupole; RSD, relative standard deviation; SFSTP, Société Française des Sciences et Techniques Pharmaceutiques; SRM, selected reaction monitoring; TOF, time-of-flight; VS, validation standard.

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1. Introduction

In clinical and forensic toxicology, a two-step methodology is generally used for the determination of drugs of abuse in biological samples. First, a rapid, sensitive and generic screening is performed, followed by an independent confirmatory procedure prior to quantitation in cases of positive results. Therefore, many analytical methods have been used for the screening and quantitation of targeted compounds, including gas chromatography (GC), liquid chromatography (LC) with mass spectrometric (MS) and tandem MS (MS/MS) detection [1–10].

Capillary electrophoresis (CE) represents an attractive alternative technique to chromatographic approaches for a wide range of clinical and toxicological applications [11–14] and has numerous advantages, such as short development and analysis times and low solvent and sample consumption [15,16].

To increase the relatively low sensitivity and selectivity encountered with conventional UV/Vis detection, CE can be hyphenated with MS. The combination of CE with a time-of-flight mass spectrometer (TOF/MS) is particularly well adapted to screening methodology due to its high mass resolution and accuracy, which allow for the identification of unknown compounds, as well as its relatively high data acquisition rate [17–20]. On the other hand, CE has also been successfully hyphenated to highly selective analyzers such as triple quadrupole (QqQ) in selected reaction monitoring (SRM) for the sensitive and selective quantitation of various xenobiotics in body fluids [21–28]. Electrospray ionization (ESI) remains the most widespread ionization source for coupling CE and MS, especially with the sheath-flow configuration [29].

The sheath-flow interface is characterized by an additional make-up liquid that mixes with the CE effluent at the capillary tip, providing electrical contact at the outlet end and the appropriate flow rate (μL range) and solvent conditions for ionization of the analytes [15,30,31]. The commercial set-up consists of two concentric tubes positioned in an orthogonal configuration toward the MS entrance. One tube surrounds the CE capillary outlet and transports the sheath liquid, and the other tube transports the nebulizing gas [29]. A new triple-tube sprayer for CE-MS coupling has been designed recently; this new sprayer should help position the sprayer needle exactly in the center of the sprayer body, improving spray quality and thus the ionization and signal intensity, enhancing the overall method sensitivity. Over the last several years, modifications of the ESI source itself as well as the sprayer position were also carried out to increase ionization and transmission efficiency, with adaptations in the position and number of heated gas inlets, the sprayer orientation relative to the sampling orifice, the diameter of MS orifice, and the number of transfer capillaries [32,33]. Some of these improvements were also envisaged for CE-MS coupling.

In CE-MS, the overall sensitivity can be further increased with the implementation of an on-line sample preconcentration (sample stacking). In case of urine analysis, the sample can be diluted and directly injected with an on-line preconcentration procedure, avoiding a tedious and time-consuming off-line sample preparation [34]. Sample stacking is performed directly in the separation capillary and is based on the velocity change of the analytes due to the field strength or pH differences between the sample zone and the running buffer [35,36]. For weak cationic compounds, mostly encountered in clinical or forensic toxicology, pH-mediated stacking can be implemented. In this configuration, a small plug of strong base (opposite pH to the background electrolyte, BGE) is injected prior to the sample. When applying the separation voltage, cationic analytes migrate through the sample until the strong alkaline plug, they become neutral and stack in a narrow zone at the boundary of the sample and the alkaline plug. Once acidified by the acidic BGE, the analytes return to their cationic state and begin the electrophoretic process [37,38]. As this procedure sharpens the sample zone, much larger sample volumes can be injected without any peak broadening.

In this study, a fast and sensitive CE-MS two-step workflow was developed for the screening and quantitation of drugs of abuse in urine samples. As a first step, a CE-ESI-TOF/MS screening method was implemented using an on-line sample preconcentration based on a pH-mediated stacking strategy to allow for a substantial loading capacity with a 10-fold urine dilution. This screening step was followed by quantitation of the compounds by CE-ESI-MS/MS with a QqQ analyzer in SRM. For this purpose, a new triple-tube sprayer and a new ESI source configuration was used. The developed CE-ESI-MS/MS method was fully validated for two model compounds, cocaine (COC) and methadone (MTD), according to protocols of the Société Française des Sciences et Techniques Pharmaceutiques (SFSTP) [39–42] and the Guidance of the Food and Drug Administration [43]. Quantitative performance was evaluated for selectivity, response function, the lower limit of quantitation (LLOQ), trueness, precision, and accuracy, and the method was eventually applied to the quantitation of COC and MTD in previously screened samples.

2. Materials and methods

2.1. Chemicals

Sodium hydroxide, analytical grade isopropanol (*i*-prOH) and 28% ammonia solution (*m*/*v*) were obtained from Fluka (Buchs, Switzerland). Methanol (MeOH), glacial acetic acid and formic acid were purchased from Biosolve (Valkenswaard, Netherlands) and were all ULC/MS grade. Ultrapure water was supplied by a Milli-Q Advantage A10 purification system from Millipore (Bedford, MA, USA). Nalbuphine hydrochloride hydrate, ketamine hydrochloride, procaine hydrochloride, trimipramine maleate, metoprolol and d-propoxyphene (D-PX) were purchased from Sigma–Aldrich (Seelze, Germany). Cocaine (COC), deuterated cocaine (d_3-COC), d,l-methadone (MTD), deuterated d,l-methadone (d_3-MTD), 3,6-diacetylmorphine (heroin), benzoylecgonine (BE), codeine, pholcodine, d,l-norephedrine, meperidine (pethidine), 6-monoacetylmorphine (6-MAM), buprenorphine, ethylmorphine, amphetamine, methamphetamine (MA), 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxamethamphetamine (MDMA), 3,4-methylenedioxyethylamphetamine (MDEA), N-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine (MBDB), norcocaine (NorCOC), ephedrine, pseudoephedrine, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and dextromethorphan in methanolic solutions (1 mg mL^{-1}) were obtained from Lipomed AG (Arlesheim, Switzerland). Cocaethylene and anhydroecgonine methyl ester (AEME) in acetonitrile (1 mg mL^{-1}) were obtained from Lipomed. Methylphenidate was a USP reference standard (Rockville, MD, USA). Fentanyl citrate was obtained from Sintetica (Mendrisio, Switzerland). The CEofix MS compatible coating kit was purchased from Analis (Namur, Belgium).

2.2. Sample preparation

2.2.1. Urine samples

Blank pooled urine was obtained from a pool of six healthy Caucasian non-drug consumers and stored after collection in polypropylene tubes at −20 °C. Before analysis, the pooled urine was defrosted at ambient temperature, centrifuged at 10,000 rpm for 5 min and filtered through a 0.45-μm nylon filter (BGB Analytik AG, Böckten, Switzerland). Stock standard solutions of the solid analytes were prepared by dissolving each compound in MeOH to obtain a concentration of 1 mg mL^{-1} and stored at 4 °C until use.

Blank pooled urine was spiked daily at desired concentrations. For this purpose, volumes of stock standard solutions were evaporated to dryness under a gentle steam of nitrogen and reconstituted in blank urine. Before injection, urine samples were diluted with BGE and water (1:1:8, *v*/*v*/*v*).

Toxicological samples were received from the Laboratory of Clinical Chemistry (Geneva Hospitals, Geneva, Switzerland) and stored at −20 °C until use. Before analysis, samples were treated in the same manner in which the blank pooled urine was treated. For COC and MTD quantitation, IS were spiked at 50 ng mL^{-1} before dilution and injection. Two independent analyses were performed for each sample ($N = 2$).

2.2.2. Solutions used for calibration

Calibration standards (CS) were independently prepared in blank pooled urine on each of the three validation series ($j = 3$) at three known concentrations (10, 500 and 1000 ng mL^{-1} for both compounds, $k = 3$) with two replicates for each concentration ($n = 2$). d_3-COC and d_3-MTD were spiked in each sample at a concentration of 50 ng mL^{-1}, and calibration curves were built from the peak areas of COC and MTD *versus* the peak areas of d_3-COC and d_3-MTD, respectively.

2.2.3. Solutions used for validation

Validation standards (VS) were independently prepared in blank pooled urine for each of the three validation series ($j = 3$) at four known concentrations (10, 25, 500 and 1000 ng mL^{-1} for both compounds, $k = 4$), with four replicates for each concentration ($n = 4$). d_3-COC and d_3-MTD were spiked in each sample at a concentration of 50 ng mL^{-1}, and reported signals were obtained from the peak areas of COC and MTD *versus* the peak areas of d_3-COC and d_3-MTD, respectively.

2.2.4. Matrix effect

The matrix effect (ME) was quantified using a method previously described by Matuszewski *et al.* [44]. Two types of samples were required. Sample A consisted of a mixture of COC- and MTD-spiked solutions at 25 ng mL^{-1} in water as a standard and was diluted with BGE and water (1:1:8, *v*/*v*/*v*) prior to injection. Sample B consisted of blank pooled urine diluted with BGE and water (1:1:8, *v*/*v*/*v*) and was spiked with a mixture of COC and MTD at 2.5 ng mL^{-1} prior to injection. The ME was calculated by comparing the peak areas of sample B *versus* sample A.

2.3. BGE

The BGE consisted of 1 M of formic acid at pH 1.8. The pH value was measured with a SevenMulti pH meter (Mettler-Toledo, Schwerzenbach, Switzerland). The BGE was prepared every four days.

2.4. Instrumentation

2.4.1. Capillary electrophoresis

CE experiments were performed with a G7100 CE system from Agilent Technologies (Waldbronn, Germany), equipped with an on-capillary diode array detector, an autosampler and a power supply able to deliver up to 30 kV. Separation was performed using a fused-silica capillary (BGB Analytik AG, Böckten, Switzerland) with a total length of 80 cm and an internal diameter of 50 μm.

For screening experiments, capillaries were coated with a commercial dynamic coating (CEofix) compatible with MS detection that was composed of an initiator and an accelerator solution. The capillary was conditioned daily with MeOH (5 min), water (5 min), 1 M NaOH (5 min), water (5 min), CEofix initiator (0.4 min with ESI source open), CEofix accelerator (0.4 min), and BGE (10 min) at 2 bar. Prior to each sample injection, the coated capillary was rinsed at 2 bar with BGE (3 min). The post-conditioning step was performed with water at 2 bar (2 min) and CEofix accelerator (1 min). After 20 runs, the coating was removed with 1 M NaOH, and the capillary was rinsed with water and recoated with CEofix initiator and accelerator with the source open. Because the coating is not fully stable at the time of first injection, the first three injections were discarded.

For quantitative experiments, an uncoated bare fused capillary was used. Prior to each sample injection, the capillary was rinsed at 2 bar with BGE (3 min).

For screening and quantitation, a preplug of 7% NH_4OH (*m*/*v*) was injected at 50 mbar for 10 s (corresponding to 0.7% of the capillary length) before hydrodynamic (HD) sample injection at 100 mbar for 150 s (corresponding to 20.5% of the capillary length), followed by a postplug injection of BGE at 50 mbar for 3 s (corresponding to 0.2% of the capillary length). Experiments were carried out in positive polarity mode (anode at the inlet and cathode at the outlet). A constant voltage of 30 kV with an initial ramping of 1667 V s^{-1} (18 s) was applied, and the capillary set at 25 °C. Samples were kept at ambient temperature in the autosampler.

2.4.2. Mass spectrometry

2.4.2.1. Time-of-flight. For screening experiments, the CE instrument was coupled to a 6210 LC/MS TOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) *via* a coaxial sheath flow ESI interface with a standard triple-tube sprayer (P/N G1607A) from Agilent Technologies. The operating parameters are summarized in Table 1. MS mass range was between 50 and 1000 *m*/*z* with an acquisition rate of 2.5 spectra s^{-1} (400 ms/spectrum, 5337 transients/spectrum).

Comparison of triple-tube sprayers was performed with the same 6210 LC/MS TOF set-up and operating parameters. MA and MTD at two concentrations (100 and 1000 ng mL^{-1}, $k = 2$), with four repetitions for each concentration ($n = 4$), were analyzed by CE-ESI-TOF/MS equipped with (i) the standard triple-tube sprayer (P/N G1607A) and (ii) the new triple-tube sprayer (P/N G1607B).

Investigations of the new ESI source (*i.e.*, Agilent Jet Stream (AJS) source) and comparison with the conventional ESI source were performed on a CE instrument coupled to a 6230 LC/MS TOF mass spectrometer (Agilent Technologies); both sources were equipped with the new triple-tube sprayer. The operating parameters are listed in Table 1. The same protocol used for the triple tube sprayers comparison was implemented: MA and MTD at two concentrations (100 and 1000 ng mL^{-1}, $k = 2$), with four repetitions for each concentration ($n = 4$), were analyzed by CE-TOF/MS with (i) the conventional ESI source and (ii) the AJS set-up.

2.4.2.2. Tandem mass spectrometry. For quantitative experiments, the CE instrument was coupled to a 6490 Triple Quadrupole LC/MS system (Agilent Technologies) *via* the coaxial sheath flow interface, and the AJS source was equipped with the new triple-tube sprayer (P/N G1607B). Table 1 presents the operating parameters. For confirmation and quantitation, the SRM transitions were fixed at *m*/*z* 304.1 → 182.0, *m*/*z* 307.1 → 185.0, *m*/*z* 310.2 → 265.1 and *m*/*z* 313.2 → 268.1 for COC, d_3-COC, MTD and d_3-MTD, respectively. The collision energy was set at 10 eV for MTD and d_3-MTD and at 20 eV for COC and d_3-COC. The dwell time and mass resolution were set at 80 ms and 0.7 u, respectively, for all transitions.

2.4.3. Software

For screening experiments, CE ChemStation version B.04.02 was used for CE control, and MassHunter version B.03.02 (both Agilent Technologies) was used for ESI-TOF/MS control, data acquisition, and data handling. For quantitative experiments, CE ChemStation version B.04.03 was used for CE control, and Mass Hunter version B.05.00 (both Agilent Technologies) was used for

Table 1
ESI-MS operating parameters.

Operating parameters	6210 TOF/MS system	6230 TOF/MS system		6490 Triple Quadrupole MS system
Source	ESI	ESI	AJS	AJS
Sheath liquid				
Composition	*i*-PrOH-water-HCOOH 50:50:0.5 (*v*/*v*/*v*)	*i*-PrOH-water-HCOOH 50:50:0.5 (*v*/*v*/*v*)		*i*-PrOH-water-HCOOH 50:50:0.5 (*v*/*v*/*v*)
Flow rate	3 μL min^{-1}	3 μL min^{-1}		5 μL min^{-1}
Nebulizing gas				
Pressure	4 psi	4 psi		8 psi
Drying gas				
Temperature	250 °C	250 °C		200 °C
Flow rate	4 L min^{-1}	4 L min^{-1}		16 L min^{-1}
Sheath gas				
Temperature	–	–	195 °C	200 °C
Flow rate	–	–	3.5 L min^{-1}	3.5 L min^{-1}
ESI				
Polarity	Positive	Positive	Positive	Positive
Voltage	+4500 V	+4500 V	+1500 V	+2000 V
Nozzle				
Voltage	–	–	+2000 V	+2000 V
Fragmentor				
Voltage	150 V	150 V		380 V
Electron multiplier				
Voltage	–	–		300 V
Cell accelerator				
Voltage	–	–		3 V

ESI-MS/MS control, data acquisition, and data handling. pK_a values were estimated using Advanced Chemistry Development (ACD/Labs) software version 11.02.

3. Results and discussion

3.1. Screening with CE-ESI-TOF/MS

3.1.1. BGE composition

Numerous drugs of abuse and respective phase I metabolites are weak basic compounds (Table 2); thus, an acidic BGE was selected to ensure their maximal ionization for proper electrophoretic mobilities, according to their charge-to-size ratio. Volatile acids such as acetic and formic acids, with or without the presence of ammonium counterions, were considered at various concentrations and pH values. TFA was discarded due to potential ion suppression during the ESI process [45,46]. The best results in terms of efficiency and selectivity for the separation of drugs of abuse were obtained with 1 M formic acid at pH 1.8.

3.1.2. Capillary coating

Using fused-silica capillaries with low-pH BGE induces a very low electroosmotic flow (EOF), which results in long analysis times that can be deleterious in clinical or forensic toxicology, where a fast screening method is required. Moreover, the variability in migration times observed in the preliminary experiments with fused-silica capillaries using direct injection of diluted urine was critical for the discrimination and identification of compounds. The use of an anionic capillary coating was therefore selected to speed up the analysis due to the formation of a high and repeatable EOF, as well as to enhance the migration times' repeatability. In this study, an MS-compatible non-covalent bilayer coating kit was used. Each step of the coating procedure was investigated by monitoring the compounds' migration times and the EOF to obtain lower variability. The capillary was first wet with MeOH, rinsed with water and then flushed with 1 M NaOH for silanol deprotonation before the capillary coating step with a polycationic (initiator) layer followed by a polyanionic (accelerator) layer. Less than one capillary volume for the initiator and accelerator was mandatory to obtain satisfactory performance. Between runs, a rinsing step with water was performed to eliminate potential urine residues before regeneration of the accelerator layer. The first three injections after the new coating were systemically discarded to stabilize the migration times. After approximately 20 runs, the coating was deteriorated, resulting in a decrease in efficiency and in the repeatability of the migration times. The capillaries were thus rinsed with 1 M NaOH to remove the coating every 20 runs before repeating the whole coating procedure. Using this coating procedure, the RSDs for the migration times were lower than 1%.

3.1.3. Injection

Fast, sensitive and generic methods are required in clinical and forensic screenings; thus, a simple dilution of the sample before injection was considered. A stacking procedure was implemented to increase the quantity injected and to offset the loss of sensitivity caused by urine dilution. In contrast to other stacking procedures, which can be strongly dependent on the saline composition of the sample, a pH-mediated stacking procedure was applied for urine analysis. Samples were 10-fold diluted with BGE and water (1:1:8, *v*/*v*/*v*) prior to injection. This dilution allowed for (i) the normalization of urine pH, (ii) a full ionization of analytes before injection, and (iii) a consequent decrease of the sample conductivity. A plug of volatile strong base was injected before the acidified urine sample. Various NH_4OH concentrations (1–14%) were tested, as well as various plug lengths. A small preplug of 7% NH_4OH (*m*/*v*), corresponding to 0.7% of the capillary length that was injected prior to the acidified diluted urine sample gave the highest analyte stacking without disrupting the electrophoretic process and without having a negative effect on the capillary coating. With the developed pH-mediated stacking procedure, 20.5% of the capillary length was filled during injection without any peak broadening. With this stacking procedure, the limits of detection (LODs), expressed as the concentration where the detected signal height was superior to 500 counts, ranged from 2 to 200 ng mL^{-1} for opiates, 2–100 ng mL^{-1} for amphetamines and derivatives, 5–50 ng mL^{-1} for COC and metabolites, and 20–50 ng mL^{-1} for various pharmaceutical and toxicological compounds in spiked urine (Table 3).

Fig. 1 shows the electropherograms obtained with the developed CE-ESI-TOF/MS screening method for blank pooled urine spiked with a set of 33 representative drugs of abuse and their main

Table 2
Physico-chemical properties of selected compounds.

	Molecular weight (Da)	m/z of detected $[M+H]^+$ (±0.005)	Basic pK_a ± $IC_{95\%}$[a]	Acidic pK_a ± $IC_{95\%}$[a]
Opiates				
Heroin	369.1576	370.1649	7.93 ± 0.40	
Morphine	285.1365	286.1438	8.25 ± 0.40	9.48 ± 0.40
Codeine	299.1521	300.1594	8.23 ± 0.40	13.40 ± 0.20
6-MAM	327.1471	328.1543	8.03 ± 0.40	9.41 ± 0.40
Ethylmorphine	313.1678	314.1751	8.24 ± 0.40	13.40 ± 0.20
Fentanyl	336.2202	337.2274	8.92 ± 0.20	
Pholcodine	398.2206	399.2278	8.22 ± 0.40	
Pethidine	247.1572	248.1645	7.84 ± 0.10	
Buprenorphine	467.3036	468.3108	8.31 ± 0.60	9.47 ± 0.60
Nalbuphine	357.1940	358.2013	7.35 ± 0.60	9.39 ± 0.60
Dextromethorphan	271.1936	272.2009	9.13 ± 0.20	
MTD	309.2093	310.2165	9.05 ± 0.50	
EDDP	277.1830	278.1903	7.71 ± 0.60	
D-PX	339.2198	340.2271	9.19 ± 0.28	
Amphetamines & derivatives				
Amphetamine	135.1048	136.1121	9.94 ± 0.10	
MA	149.1204	150.1277	10.38 ± 0.10	
MDA	179.0946	180.1019	9.94 ± 0.10	
MDMA (ecstasy)	193.1103	194.1176	10.32 ± 0.10	
MDEA	207.1259	208.1332	10.34 ± 0.19	
MBDB	207.1259	208.1332	10.46 ± 0.20	
Ephedrine	165.1154	166.1226	9.38 ± 0.10	
Pseudoephedrine	165.1154	166.1226	9.38 ± 0.10	
Norephedrine	151.0997	152.1070	8.47 ± 0.10	
Methylphenidate	233.1416	234.1489	9.51 ± 0.10	
COC & metabolites				
COC	303.1471	304.1543	8.97 ± 0.60	
Cocaethylene	317.1627	318.1700	9.04 ± 0.60	
AEME	181.1103	182.1176	7.97 ± 0.40	
NorCOC	289.1314	290.1387	9.02 ± 0.60	
BE	289.1314	290.1387	10.83 ± 0.40	
Others				
Metoprolol	267.1834	268.1907	9.43 ± 0.10	
Procaine	236.1525	237.1598	9.24 ± 0.25	
Ketamine	237.0920	238.0993	6.46 ± 0.20	
Trimipramine	294.2096	295.2169	9.38 ± 0.28	

[a] Calculated using ACD/Labs software version 11.02.

phase I metabolites. This screening procedure appears to be well suited for applications in clinical or forensic toxicology due to (i) its speed, with less than 15 min required for the whole analytical procedure (including sample pretreatment, capillary pre-conditioning, analysis, and post-conditioning); (ii) its application to a wide set of toxicological or forensic basic compounds with a simple urine dilution; (iii) its high sensitivity due to the on-line sample preconcentration, allowing for LODs as low as to 2 ng mL^{-1}; and (iv) its high selectivity, where CE enables the separation of isobaric compounds and TOF/MS enables the discrimination of co-migrating analytes.

3.2. Quantitation by CE-ESI-MS/MS

As a second step of the toxicological or forensic procedure, a highly sensitive and accurate confirmatory method is required to confirm or quantify compounds that have been positively identified by the screening method. In this context, CE was hyphenated to a highly sensitive QqQ mass analyzer equipped with a completely novel source configuration, composed of a new triple-tube sprayer and a new ESI source. The new triple-tube sprayer has a modified design consisting of a revised needle length and tip shape that help the needle position itself exactly in the center of the sprayer body

Table 3
LODs expressed with signal heights above 500 counts.

Opiates	LOD (ng mL^{-1})	Amphetamines & derivatives	LOD (ng mL^{-1})	COC & metabolites	LOD (ng mL^{-1})
Heroin	20	Amphetamine	2	COC	10
Morphine	20	MA	50	Cocaethylene	10
Codeine	50	MDA	2	AEME	10
6-MAM	50	MDMA (ecstasy)	10	NorCOC	20
Ethylmorphine	50	MDEA	10	BE	50
Fentanyl	20	MBDB	10	Others	LOD (ng mL^{-1})
Pholcodine	200	Ephedrine	50		
Pethidine	2	Pseudoephedrine	50	Metoprolol	20
Buprenorphine	50	Norephedrine	100	Procaine	20
Nalbuphine	50	Methylphenidate	10	Ketamine	50
Dextromethorphan	20			Trimipramine	50
MTD	20				
EDDP	10				
D-PX	20				

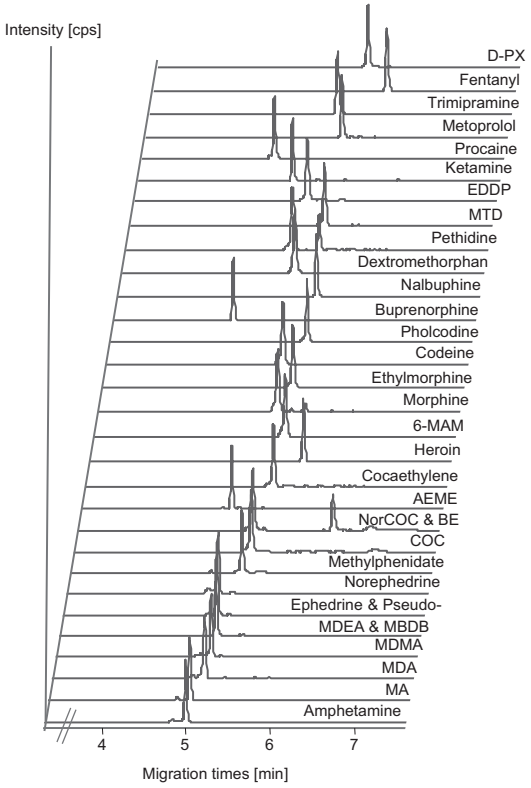

Fig. 1. Extracted ion electropherograms (±0.005 Da) of 33 compounds in urine at 100 ng mL^{-1}. See text for experimental conditions (Section 2.4).

and that should thus improve the spray quality, and potentially ionization and signal intensity. The new ESI source is characterized by the addition of a collinear, concentric superheated nitrogen sheath gas surrounding the sprayer that confines the spray plume and thus increases the desolvation efficiency. Due to an enhanced ion density in the confinement zone, a higher number of ions are available for sampling, and the overall ionization should be more efficient [47].

To evaluate the impact of these geometric modifications on CE-MS analyses, the new triple-tube sprayer was first compared to the standard sprayer prior to the evaluation of the performance of the new ESI source.

3.2.1. Evaluation of triple-tube sprayers and ESI sources

The ionization efficiency of the standard triple-tube sprayer was compared to that of the new triple-tube sprayer with two model compounds, MA and MTD, at various concentrations, with the conventional ESI source. The signal intensities, expressed by peak heights, were found to increase by a factor 1.5 with the new triple-tube sprayer. This increase in sensitivity was also confirmed with other copies of the new sprayer.

Comparison of the new ESI source *versus* the conventional one, both equipped with the new triple-tube sprayer, was performed after the determination of the operating parameters. It has to be noticed that relatively low sheath gas temperature and flow rate values (*i.e.*, 195 °C and 3.5 L min^{-1}, respectively) were selected due to the very low flow rate (composed of CE effluent and sheath liquid flow rate) coming out of the sprayer tip. Higher sheath gas temperatures and flow rate values that are recommended for LC-MS configuration were found deleterious for CE-MS coupling. The influence of this sheath gas on the migration times was also considered regarding a potentially enhanced suction (siphoning) effect. No significant difference was observed with the new source, probably due to its distance with the capillary tip.

The signal intensities, expressed by peak heights for MA and MTD were found to be significantly lower for the new ESI source and were explained by the relatively high value of the sheath gas flow rate which was selected due to instrumental constraints. Nevertheless, the use of isotopic IS correction was selected for quantitative purposes in CE-MS to lower the matrix effects (MEs) and to correct the lack of repeatability during injection. As a result, relative compound areas with deuterated IS correction did not show any significant difference between the two ESI sources for MA and MTD. Therefore, similar performance was observed for the new CE-MS configuration, *i.e.*, new triple-tube sprayer in combination with new ESI source, compared to the conventional set-up.

3.2.2. CE-ESI-MS/MS method development

Among the numerous illicit drugs that may be illegally obtained, COC consumption has been increasing over the last years; currently, it is one of the most common drugs detected in toxicological samples. Meanwhile, an increase in the consumption of MTD has been observed for opioid substitution therapy and pain relief. COC and MTD were thus selected as representative model compounds to illustrate a full quantitative procedure in urine samples by CE-ESI-MS/MS with QqQ in SRM mode. The same BGE and injection conditions, *i.e.*, urine dilution and pH-mediated stacking procedure, were used. With these separation conditions, the repeatability of the migration times was acceptable with RSDs between 3% and 10%, as for the intermediate precision with RSDs from 7% to 8%. The transitions were determined by compound infusion, and the most sensitive and specific transitions were selected for confirmation as well as quantitative purposes, *i.e.*, m/z 304.1 → 182.0, m/z 307.1 → 185.0, m/z 310.2 → 265.1 and m/z 313.2 → 268.1 for COC, d_3-COC, MTD and d_3-MTD, respectively. With these conditions, LODs (expressed as the concentration where signal-to-noise ratio was superior to 3) were estimated at 2 ng mL^{-1} for MTD and COC, which was considered an appropriate value for a simple injection of diluted samples without off-line sample preparation.

3.2.3. Selectivity

With urine dilution, endogenous compounds that may alter the analyte ionization process (signal suppression or enhancement) are still present. ME was therefore evaluated for COC and MTD to determine the influence of potential co-migrating interferents on analyte ionization prior to QqQ determination. For this purpose, a procedure based on Matuszewski *et al.* [44] for the quantitation of MEs in biological fluids was implemented. COC did not show any significant suppression, with an ME of 92 ± 9% (±2SD), while a relevant signal suppression was observed for MTD, with an ME of 73 ± 5%. Therefore, the use of deuterated IS correction for an external calibration within the reconstituted matrix was selected for quantitation.

The method selectivity was also evaluated by comparing electropherograms obtained by injecting (i) blank urines (CAL 00), (ii) blank urines spiked with d_3-COC and d_3-MTD at 50 ng mL^{-1} (CAL 0) and (iii) a VS at 25 ng mL^{-1} for COC and MTD, with the IS set at 50 ng mL^{-1}. As illustrated for a blank urine in Fig. 2, no interference was detected at the migration times corresponding to COC and MTD or their respective IS.

3.2.4. Validation

SFSTP validation guidelines [41] were followed to evaluate the quantitative performance of the developed CE-ESI-MS/MS method for COC and MTD analysis on three independent series (j = 3). The validation protocol was adapted from protocol V5, which recommends a calibration within the matrix using a minimum of two repetitions (n = 2) for CS at least for three concentrations (k = 3),

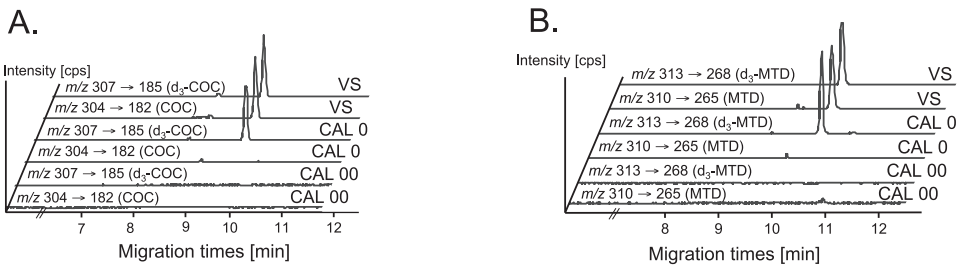

Fig. 2. Evaluation of the method selectivity. (A) Electropherograms obtained for COC by injecting blank pool urine (CAL 00), pooled urine spiked with d_3-COC at 50 ng mL^{-1} (CAL 0) and pooled urine spiked with COC at 25 ng mL^{-1} and d_3-COC at 50 ng mL^{-1} (VS). (B) Electropherograms obtained for MTD by injecting blank pool urine (CAL 00), pooled urine spiked with d_3-MTD at 50 ng mL^{-1} (CAL 0) and pooled urine spiked with MTD at 25 ng mL^{-1} and d_3-MTD at 50 ng mL^{-1} (VS). See text for experimental conditions (Section 2.4).

and a minimum of three repetitions ($n = 3$) for VS at least for three concentrations ($k = 3$) [41]. In this context, the validation protocol involved three concentrations ($k = 3$) with two repetitions ($n = 2$) for CS and four concentrations ($k = 4$) with four repetitions ($n = 4$) for VS. The concentrations' ranges for COC and MTD were determined according to the standard concentrations detected in samples from drug consumers. For COC, urine concentration depends on the route of administration (intravenous, intranasal, smoking route), the dose, and the time of urine collection (elimination half-life of *ca.* 2–4 h) [48,49]. MTD urine concentration relies on oral dose, enantiomer *versus* racemate administration, cytochrome P450 metabolism intervariability and the time of urine collection (elimination half-life of *ca.* 23 h) [50,51]. Therefore, a large concentration range was selected, from 10–1000 ng mL^{-1}, for both compounds.

CS and VS were prepared in blank pooled urine. Several regression models for calibration curve adjustment were evaluated. Trueness (relative bias) and precision were assessed for each concentration level. Precision was estimated with the variances of repeatability (s_r^2) and intermediate precision (s_R^2) and was expressed by RSD (%). Confidence intervals were calculated with fixed degrees of freedom ($df = k \times j - n$) at a risk $\alpha = 5\%$.

3.2.4.1. Response function. Different regression models were assessed for the calibration curve, including ordinary least square (OLS) regression, OLS after square root transformation of concentrations (x) and responses (y), OLS after logarithm transformation of concentrations (x) and responses (y), OLS forced through the origin, external standard with high level of CS and weighting least square with two weighting factors ($1/x$ and $1/x^2$). For all of the calibration models, accuracy profiles were plotted for COC and MTD. The optimal regression model was selected according to the best total error profile obtained when covering the whole concentrations' range. OLS after square root transformation was selected as the best calibration model for COC with confidence intervals contained inside of the acceptance limits, which were set at ±30% according to the guidelines for quantitation in bioanalysis [52]. For MTD, the best model for calibration was the OLS after square root transformation, but the lowest VS (10 ng mL^{-1}) was outside of the acceptance limits. The limit of quantification was therefore established on the basis of the accuracy profile, as discussed below.

3.2.4.2. Trueness and precision. The trueness of an analytical procedure expresses the closeness of agreement between the mean values obtained from a series of measurements and the true values [41]. The results for trueness were assessed from the VS by relative bias [%] and are presented in Table 4. For COC, the relative biases were all satisfactory, as they did not exceed the threshold of ±15%. For MTD, the relative biases for medium and high concentrations, *i.e.*, 25, 500 and 1000 ng mL^{-1}, were lower than ±2%. However, with a relative bias of 38.5%, the lowest concentration (10 ng mL^{-1}) was unacceptable.

The precision of the method was estimated using the repeatability and intermediate precision at each VS and was expressed by RSDs. As presented in Table 4, the RSD values for COC were in the range of 3.0–5.7% for both repeatability and intermediate precision, showing strong precision in the developed method. For MTD, unsatisfactory RSD values of 21.0 and 27.8% were obtained at 10 ng mL^{-1} for repeatability and intermediate precision, respectively, while the RSDs were lower than 7.1% for higher concentrations.

3.2.4.3. Accuracy. Accuracy is the expression of the total error of the analytical method and was chosen to evaluate the capacity of the developed analytical method to quantify samples with an accepted risk of $\alpha = 5\%$ [43,53]. The lower and upper confidence limits of the mean bias (%) for COC, as shown in Table 4, were included within the acceptance limits of ±30% for each level of concentration. The developed method is therefore accurate for the quantitation of COC over the investigated concentration range (10–1000 ng mL^{-1}). The lowest concentration level (10 ng mL^{-1}) was confirmed to be the LLOQ, which is defined by the smallest quantity of analyte that can be quantified with a defined accuracy within the acceptance limits.

For MTD, the LLOQ was interpolated from the absolute accuracy profile of MTD and defined at 21 ng mL^{-1} because the lowest concentration level (10 ng mL^{-1}) was not included within the acceptance limits of ±30%. With this LLOQ, the quantitation of MTD was found to be accurate in the range of 21–1000 ng mL^{-1}.

Table 4
Validation criteria and results for COC and MTD in urine ($j = 3$, $k = 4$, $n = 4$).

Validation criterion	COC	MTD
Trueness		
Relative bias (%)		
10 ng mL^{-1}	12.0	38.5
25 ng mL^{-1}	1.0	1.7
500 ng mL^{-1}	−3.1	−1.8
1000 ng mL^{-1}	0.1	0.7
Precision		
Repeatability/intermediate precision (RSD, in %)		
10 ng mL^{-1}	5.7/5.7	21.0/27.8
25 ng mL^{-1}	5.0/5.0	7.1/7.1
500 ng mL^{-1}	4.1/4.1	2.8/3.6
1000 ng mL^{-1}	3.0/3.3	3.0/3.0
Accuracy		
Lower/upper confidence limits of the total errors (%)		
10 ng mL^{-1}	−1.1/25.1	−25.5/102.5
25 ng mL^{-1}	−10.5/12.4	−14.7/18.0
500 ng mL^{-1}	−12.5/6.3	−10.1/6.5
1000 ng mL^{-1}	−7.4/7.6	−6.2/7.6
LLOQ (ng mL^{-1})	10	21

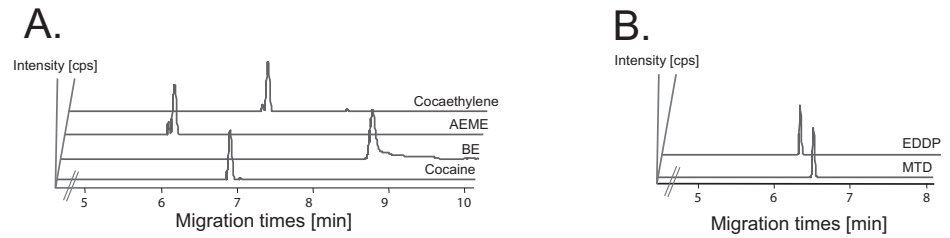

Fig. 3. Extracted ion electropherograms (±0.005 Da) obtained for positive urine samples in screening analysis. (A) COC and metabolites (sample #1). (B) MTD and metabolite (sample #2). See text for experimental conditions (Section 2.4).

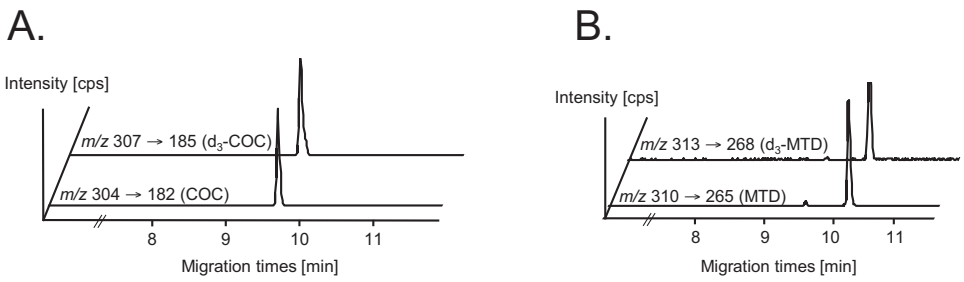

Fig. 4. Electropherograms obtained for toxicological samples in quantitative analysis. (A) Sample #1 containing COC, (B) Sample #2 containing MTD. See text for experimental conditions (Section 2.4).

3.3. Application to real samples

The complete two-step CE-MS methodology was eventually applied for the analysis of urine samples collected from drug consumers and received from the Laboratory of Clinical Chemistry (Geneva Hospitals, Geneva, Switzerland). Samples were first analyzed with the developed screening CE-ESI-TOF/MS method. Identification of the drugs was achieved (i) by comparison of migration times with spiked standards in blank urines, (ii) with accurate mass, and (iii) with the detection of phase I metabolites. Two of the samples were selected on the basis of the identification of COC and MTD. The obtained electropherograms are shown in Fig. 3. In the first sample (Fig. 3A), COC and its specific metabolite BE were detected, as well as cocaethylene, which is produced by the concomitant consumption of COC and ethanol, and AEME, which can be detected in cases of smoked COC. In the second sample (Fig. 3B), MTD and its specific metabolite, EDDP, were detected. The detection of various phase I metabolites was highly useful for confirming the identification of drugs of abuse and providing complementary and valuable information about co-consumptions.

The COC and MTD detected in the positive samples were then quantified with the developed CE-ESI-MS/MS method. A calibration curve was constructed the same day ($k=3$, $n=2$), and OLS was applied after square root transformation of concentrations and responses. The confidence interval associated to the mean was expressed with:

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

where $\bar{x}$ is the mean result and N is the number of analyses. $t_{df,\alpha}$ (Student constant dependent on α and df), s_r^2 and s_g^2 were determined during a validation with regular ANOVA-based variance decomposition. Because most of the variability came from repeatability (s_r^2) and not from the interseries variance (s_g^2), two replications ($N=2$) were performed to reduce the intra-day variability and to obtain a narrow confidence interval for the final result. In the first sample, COC concentration was found to be $41.0 \pm 6.4\,\text{ng mL}^{-1}$. The corresponding electropherograms are presented in Fig. 4A. This relatively low concentration can be related to a low dose (*e.g.*, less than *ca.* 10 mg of crack, intranasal, or intravenous dose) and/or a late urine collection (>24 h) after COC consumption [54]. In the second sample, $462.9 \pm 33.5\,\text{ng mL}^{-1}$ of MTD were detected. The electropherograms obtained for the second sample are illustrated in Fig. 4B. Due to the relatively long detection time window of MTD in urine and the high CYP450 inter-individual variability [55,56], this concentration can be related to both initial and maintenance MTD treatment.

4. Concluding remarks

A fast and sensitive CE-ESI-MS two-step workflow was developed for the screening of drugs of abuse in urine samples prior to their quantitation. A CE-ESI-TOF/MS method was implemented for the screening step with a pH-mediated stacking procedure, which avoided a tedious off-line sample preparation with a simple urine dilution. The higher loading capacity (more than 20%) led to an increased sensitivity while maintaining strong efficiencies. The CE-ESI-TOF/MS method allowed for LODs as low as $2\,\text{ng mL}^{-1}$ for a varied set of common drugs of abuse and pharmaceutical compounds.

The screening step was followed by compound quantitation by CE-ESI-MS/MS with a QqQ analyzer equipped with a new ESI source and a new triple-tube sprayer design, which did not show significant differences compared with the conventional ESI source and sprayer. The quantitative procedure was fully validated for COC and MTD according to reference guidelines based on selectivity, response function, trueness, precision, and accuracy. COC analysis was found to be accurate over the range of 10–1000 ng mL^{-1}, with accuracy included within the ±30% tolerance limits, and MTD analysis was accurate in the concentration range of 21–1000 ng mL^{-1}.

The developed two-step strategy was eventually applied to the analysis of real cases and was found to be applicable for a fast and sensitive screening as well as for accurate quantitation in urine samples.

Conflict of interest

The authors have no conflicts of interest to declare.

Acknowledgments

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Chapter IV.

Chapter IV. Contribution of CE-MS for intact protein analysis

1 Protein analysis

In toxicology, the vast majority of the targeted compounds are low-molecular weight xenobiotics presenting a mass lower than 1 kDa. Nevertheless, some clinical or forensic applications involve the determination of endogenous proteins, such as erythropoietin (EPO) or hemoglobin in doping control, or numerous biomarkers for diagnostic purpose, e.g., albumin for kidney perturbations, troponin I for cardiovascular diseases, C-reactive protein for inflammatory events, or β- and γ-globulins in case of lymphoproliferative diseases [1-3]. Due the much complex structure of proteins and the large differences compared to low-molecular weight compounds, alternative strategies are implemented for the analysis of proteins.

1.1 Protein analysis by mass spectrometry

The analysis of proteins by MS generally relies on two strategies, referred to as *bottom-up* and *top-down* approaches. A bottom-up analysis involves the proteolytic digestion of purified protein(s) (*e.g.*, by LC or gel electrophoresis) or complex protein mixtures leading to the formation of numerous peptide products which are analyzed by MS (*e.g.*, IT, QqQ, TOF, QTOF, Orbitrap©, *etc.*) [4,5]. By comparing the obtained peptide mass spectra with those predicted from a sequence library in a peptide spectral library, the identity of the original protein(s) can be determined. The identification process follows two strategies, *i.e.*, (i) peptide mass fingerprinting, where the peptide masses obtained by HRMS are compared to predicted, *in silico*-generated fragmentation pattern of the investigated peptides; or (ii) MS-MS experiments, where a peptide ion is selected in the first quadrupole Q1 and fragmented by CID; followed by the determination of the aminoacid sequence of the precursor ion from the masses of the fragmented ions [5,6]. The bottom-up approach is the most mature and widely used strategy in proteomics for protein identification and characterization. Numerous control software and bioinformatics tools are commercially available to help in the data treatment. However, this approach presents some limitations, including the partial protein sequence coverage by identified peptides, the loss of information about post-translational modifications (PTMs) such as phosphorylation or glycosylation which are often clinically relevant, and a long analysis time for very complex peptide mixtures [5,6]. Furthermore, the technique relies on the hypothesis that the proteolytic cleavage (most frequently carried out with trypsin) is specific and repeatable, allowing for an accurate identification. However, this is not always the case with a number of produced peptides often higher than the predicted amount due to artifacts or residual products, which can hinder the identification in case of very low concentrated proteins.

In the top-down approach, proteins are first separated under their intact form, *e.g.*, by using gel-based methods such as two-dimensional polyacrylamide gel electrophoresis or gel-free techniques. Subsequent to ESI or MALDI ionization process, the intact protein ions are fragmented by CID, electron capture dissociation, or electron transfer dissociation prior to their detection [5]. The two latter dissociations are preferred due to the preservation of PTMs during the fragmentation process, and provide a high sequence coverage complementary to that of CID [6]. Top-down MS approaches

lead to important and complementary information on the structure of the protein, a better characterization of the PTMs than the bottom-up approach, and allow for protein quantitation [4,5].

The gel-free techniques such as LC or CZE combined with MS, most frequently *via* ESI source, can also be considered for the determination of intact proteins which are analyzed and detected under the intact form. Since no fragmentation occurs in the context of intact protein analysis, high resolution mass analyzers such as TOF instruments are conventionally used.

Due to its high speed and high resolving power, LC is recognized as a promising analytical tool for intact protein analysis. Various LC modes of separation including RPLC, ion-exchange chromatography (IEC), size-exclusion chromatography (SEC), HILIC, and affinity chromatography can be considered [4,7]. RPLC is readily compatible with MS detection but suffers from protein adsorption, carryover, low retention, multiple peak formation due to the different conformations or PTMs, and low chromatographic performance [4,8]. These relevant drawbacks can be overcome by using wide-pore core-shell particles, fully porous *sub*-2 µm particles, organic monoliths, porous layer open tubular columns, or an elevated temperature which can all enhance the separation efficiency and/or the selectivity [8,9]. These approaches are increasingly considered in the analysis of biopharmaceuticals (*i.e.*, pharmaceuticals produced by biotechnology, encompassing recombinant proteins or monoclonal antibodies) for protein characterization, stability studies, impurity profiling, or determination of aggregates [4,9,10]. SEC, which separates proteins according to their molecular sizes, and IEX, whose separation relies on the difference in charges, are widely used but their MS coupling remains complex due to the high salt concentration present in the mobile phase [4]. All LC approaches share the similar issue encountered with protein adsorption, which does not only occur in the chromatographic column, but also in the whole instrument. It is thus important to use inert materials such as stainless steel or titanium instead of the conventional polyether ether ketone for tubing and injection needles, or used dedicated commercially available LC system for biomolecules analysis [4].

Providing several advantages in terms of efficiency and selectivity, CE-based techniques show a growing interest for intact protein analysis. Numerous CE modes, including CZE, CGE, CIEF, CEC, and less frequently MEKC or MEEKC have been used in multiple applications. Due to the relative simplicity in its combination with MS, CZE is considered attractive and has already shown it applicability for intact protein analysis, including biopharmaceuticals characterization, as well as biomarker discovery, PTMs determinations, or metalloproteins analysis in biological samples [11-15]. Both ESI and MALDI can be used to hyphenate CZE to MS. MALDI/TOF offers the advantage to give rapid information on the molecular mass of the protein and is less sensitive to buffer agents, salts, and denaturants present in the sample; but its on-line coupling remains tedious. ESI is the primary choice for on-line coupling of CZE to MS and, due to the production of multicharged ions, it can be used with conventional MS instruments presenting a relatively low mass range [14].

1.2 Protein adsorption

As the diffusion coefficients of proteins are relatively small, the separation efficiencies obtained in CZE are expected to be outstanding with a theoretical plates number up to 10^6 or more, according to **Eq. 1.14**. Moreover, a high selectivity between proteins or isoforms is anticipated due to the large

differences in their charge-to-size ratios. Nevertheless, the quality of the separation is often hampered by the adsorption process which is related to the inherent tendency of intact proteins to adhere onto the fused-silica surface of the capillary [11,16,17].

1.2.1 Adsorption phenomenon

The adsorption process is mainly driven by electrostatic interactions, dipole-dipole interactions, dispersion, and hydrogen bonds, as well as structural changes in the protein. The interactions are not only occurring with the silica wall but also with the BGE components, water, or the modified surface of the capillary in the interfacial region [14,17-20]. Once adsorbed, a protein might undergo a partial unfolding which serves as a new site for further protein attachment. The structural changes of the protein are also inherent to the BGE composition (concentration of electrolytes, pH) and can lead to peak-broadening due to conformational changes or the existence of multiple conformations.

The adsorption process can be either reversible or irreversible, as illustrated in **Fig. 4.1**. After transportation of the protein to a stagnant layer adjacent to the capillary wall within milliseconds or microseconds, the second step involves the interaction with the silica surface and the interfacial region. The protein attachment, occurring when the protein surmounts an energetic barrier, can lead to a perturbation of its structure. However, even with a change of its conformation, the protein can be reversibly desorbed from the surface (*reversible adsorption*), especially if the residence time is short. Alternately, the protein may relax into an irreversible steady-state change of conformation, leading to a tight retention to the surface (*irreversible adsorption*) [17,18].

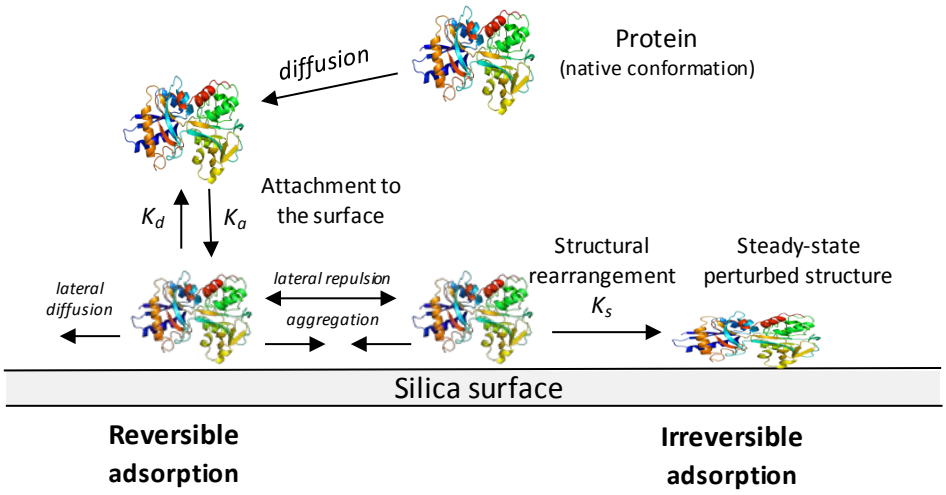

Figure 4.1. Adsorption process onto the silica surface. Adapted from [17] and [18].

This relative complex process is influenced by multiple parameters, including the protein properties, the BGE composition and pH, the temperature, the nature of the solid surface and its treatment history (*e.g.,* conditioning, rinsing, or coating procedures), and the presence of potential additives. Adsorption is a non-specific process which passes through a series of quasi-equilibrium states [17]. Hydrophilic aminoacids are usually located on the periphery of the protein and can electrostatically interact with the negatively charged silanols (mainly occurring at high pH, or with residual silanols at low pH) or with other proteins present in the environment. Knowing the isoelectric point p*I* of a protein can help predict the protein behavior; however, even with a negative global charge, a protein

can be attracted to the silica surface by electroattraction of positive patches on the protein surface or due to conformational changes as well as other hydrophobic interactions [18]. Moreover, the presence of co- and counter-ions of the BGE highly influences the net charge of both protein and silica surface due to the modification of the ξ potential, as explained in **Chapter I**, which also modifies the interactions. Finally, the proteins comprise a heterogeneous structure with predominantly hydrophilic but also a minor extent of hydrophobic regions which depend on the composition of the environment [17]. Therefore, the prediction of protein adsorption and the related interactions remains very complex. This is not only true in case of single protein analysis but remarkable for protein mixtures, involving additional mechanisms of competitive adsorption. It has been shown that adsorption is dominating for small proteins in a mixture composed of large and small proteins, whereas the adsorption of large molecules later occurs and is accompanied by the displacement of the previously adsorbed small proteins [17].

Numerous criteria can be used to measure the protein adsorption. Reversible adsorption can be evaluated by monitoring the peak efficiency (plates number) and the protein mobility (repeatability of the migration time). The evaluation of irreversible adsorption includes the measurement of protein recovery, modification of EOF mobility, and shift change in baseline detection [18,21]. Protein recovery can be assessed by measuring the loss of protein encountered within the capillary (decrease in peak area) following a methodology proposed by Towns and Regnier [22]. This procedure involving the use of two UV detectors and, thus, a custom CE instrument, improved methods have been later proposed by Lucy and coworkers [23] and more recently by Espinal *et al.* based on the hypothesis that the protein recovery exponentially decreases as a function of the migrated distance [24]. These methods allow for determining the protein recovery in conventional CE instruments. Dedicated devices may also be used to study the protein recovery, as illustrated by Staub *et al*. who studied the irreversible adsorption with an ActipixTM D100 U Area Imaging System (Paraytec, York, UK) allowing for two passes of the capillary through the UV detector, and, thus, the assessment of the decrease in area between the first and the second pass [21].

Although being a complex phenomenon and difficult to predict, multiple strategies can be considered to significantly lower the protein adsorption. More specifically, the stabilization of successive analyses relies on (i) an effective prevention of protein adsorption by appropriate selection of the separation conditions and (ii) the effective protein desorption between individual runs by adapted rinsing procedures. Even by combining multiple strategies, it is worth mentioning that a complete suppression of protein adsorption remains impossible, especially in complex analytical situations (*e.g.*, complex matrices, protein mixture, *etc.*) [17].

1.2.2 Strategies to reduce protein adsorption

1.2.2.1 Modification of separation conditions

Simple between-runs rinsing procedures with BGE or 0.1 M NaOH solution are not always efficient. An increase in NaOH concentration in the rinsing solution (or NH_4OH in case of MS detection) can lead to an enhanced desorption, but it often remains insufficient in case of strong adsorption. In these cases, high concentrated hydrochloric or phosphoric acids may give better results.

Besides the rinsing procedures, numerous strategies can be considered when selecting the separation conditions for efficient adsorption prevention, *i.e.*, (i) use of extreme pH, (ii) use of phosphate-based BGEs, (iii) use of high-ionic strength BGEs, (iv) addition of organic solvents to the BGE, (v) addition of surfactants to the BGE, (vi) addition of small molecules (*e.g.*, amines) to the BGE, and (vii) capillary coating [14,17,18,21,25-29]. By modifying the electrostatic and hydrophobic interactions encountered between the protein-BGE-surface system, and/or changing the conformation of the protein, these strategies can prevent the protein adsorption. All of these approaches except the use of phosphate-based buffers and surfactants may be employed in CE-MS coupling. Careful investigations are required to find the best compromise between adsorption prevention and system stability as well as maintain acceptable performance, mainly when using high ionic strength BGEs or small molecule additives due to an increased Joule heating effect.

Among the listed strategies, the use of capillary coatings likely remains mostly efficient for the analysis of a wide range of proteins and is extensively used in CE-MS for intact protein determination [11-13,30-35].

1.2.2.2 Capillary coating

The principle of capillary coating was briefly presented in the **Section 2.2.1** of **Chapter I**. On the contrary of low-molecular weight compounds, where the capillary coating can be empirically selected according to the physico-chemical properties of the targeted compounds and the operating conditions, the selection of the most appropriate coating for protein analysis is tedious. As previously discussed, the adsorption phenomena are hardly predictable and, thus, the coating selection is not trivial. Moreover, coating the silica surface with agents induces the modifications of the separation conditions, including (i) a sterically masking of the residual silanol groups, and (ii) an increased viscosity at the boundary of the capillary wall to hinder the accessibility to the protein, which contribute to the difficulty in predicting the adsorption mechanisms. Therefore, for analysis of a single protein by CE-MS, it is recommended to practically test as many static-adsorbed and/or static-covalent coatings as possible in case of intact protein analysis by CE-MS.

Static-adsorbed coatings are usually obtained by simply flushing the capillary with one or more polymeric solution(s) which are physically adsorbed to the fused-silica surface through forces similar to those encountered with protein adsorption. These coatings present interesting advantages compared to the covalent ones, *i.e.*, (i) the simplicity and rapidity of the coating formation (usually directly in the CE instrument), (ii) the possibility of coating regeneration, and (iii) the access to the polymer properties [18]. Depending on the charge of the flushed polymers, static-adsorbed coatings may be neutral or positively/negatively charged and consist in one or multiple successive layers of positive and negative polymers. The use of multiple successive layers coating is referred to as SMIL coatings (*successive multiple ionic-polymer layers*) and provide a substantial enhancement of coating stability compared to the single layer coatings due to the considerable higher interactions between the polymeric layers than the forces prevailing between polymers and silica wall [17]. The last layer is used to categorize the coating as *anionic*, *neutral*, or *cationic*. **Fig. 4.2** illustrates a SMIL procedure to obtain a bilayer cationic coating.

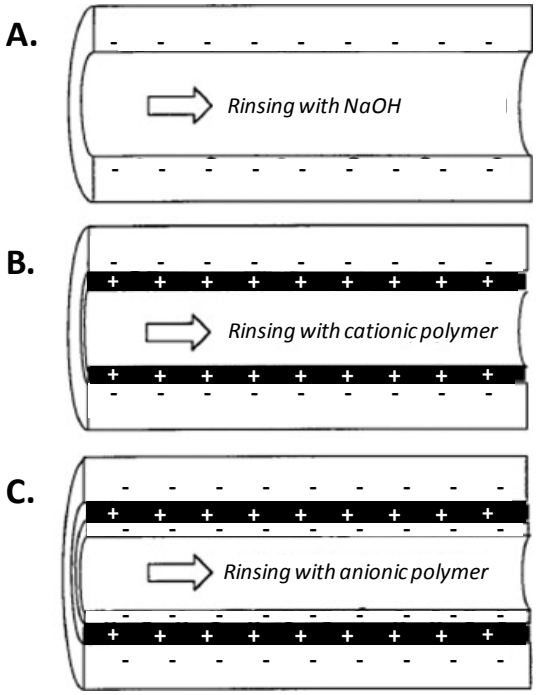

Figure 4.2. Illustration of SMIL coating procedure. The first step of the procedure involves the activation (deprotonation) of the silanol groups by rinsing the capillary with NaOH (A.). A solution containing a cationic polymer is then flushed to adsorb the first layer of the coating (B.). The second and last layer is obtained by rinsing the capillary with a solution containing an anionic polymer (C.). Adapted from [18].

In **Article IV**, numerous static-adsorbed coatings, which are frequently used in intact protein analysis, are presented and were tested to prevent the adsorption of transferrin (Tf). Tf is one of the most abundant serum proteins and is used in clinical or forensic purposes for the determination and the monitoring of chronic alcohol consumption.

2 Carbohydrate-deficient transferrin

According to the National Council on Alcoholism and Drug Dependence and the American Society of Addiction Medicine, the *alcoholism* can be defined as a primary, chronic disease with genetic, psychosocial, and environmental factors influencing its development and manifestations. It is characterized by impaired control over drinking, preoccupation with alcohol, use of alcohol despite adverse consequences, and distortions in thinking [36]. Depending on the authors, a regular consumption of *ca.* ≥ 60-80 g of ethanol *per* day is recognized as alcohol abuse [37,38]. The *chronic* abuse is encountered in case of daily consumption and does thus not (yet) consider other drinking habits such the so-called *binge-drinking* (periods of heavy drinking followed by abstinence) or other sporadic drunkenness [39].

Due to the physiological and psychological risks as well as the high prevalence of co-morbidities associated to the chronic alcohol consumption, its early diagnostic and a continuous monitoring are important and applied in numerous clinical and forensic fields, *e.g.*, occupational medicine, drunk driving, or in case of license reapplication. Besides the clinical evaluation, the diagnosis of alcohol abuse relies on the determination of (i) direct markers, such as EtG or phosphatidylethanol, which derive from alcohol metabolism, and (ii) indirect markers, such as aspartate aminotransferase

(ASAT), alanine aminotransferase (ALAT), γ-glutamyltransferase, and carbohydrate-deficient transferrin (CDT) which reflect the toxic effect of ethanol on tissues and biochemical pathways [40,41].

ASAT, ALAT, and GGT are all hepatic enzymes, and Tf is synthesized in the liver. Therefore, these markers are prone to modifications in case of chronic alcoholism due to the large effect of ethanol on the liver functions. The liver is not only the place where protein synthesis occurs but also PTM processes such as phosphorylation and glycosylation. Glycosylation it the most common PTM and consists of the addition of glycans to the protein into the endoplasmic reticulum (ER) to form oligosaccharide chains. Two types of glycosylation can occur, *i.e.*, *N*-glycosylation to the amide of asparagine side chains and *O*-glycosylation to the hydroxyl groups of serine or threonine side chains [42]. *N*-glycosylation is more prone to modifications during the glycosylation process. The latter is occurring in the ER and Golgi apparatus and consists of the formation of an oligosaccharide-lipid complex containing glucose, *N*-acetylglucosamine, and mannose, which is then transferred to a growing polypeptide with simultaneous removal of three glucose residues and one mannose. The lipid portion (dolichol) acts as a carrier molecule. This pre-protein migrates to the Golgi apparatus for further residue removal until forming a heptasaccharide, which may be then modified by glycosyltransferases and glycosidases [42]. The glycosylation is important in many biological processes such as molecular recognition, protein folding, solubility, stability, or half-life in blood, and concerns almost all endogenous proteins, except albumin and C-reactive protein [42,43].

It has been widely accepted that in all major liver diseases, changes in the protein glycosylation will occur, which is also the case for chronic alcohol consumption. Some proteins have already shown to be prone to modifications in their glycosylation process under alcohol consumption, such as apoprotein E [44], orosomucoid, α1-antitrypsin, ceruloplasmin [45], and haptoglobin [46], but only Tf is used for chronic alcohol diagnostic *via* CDT determination, mainly due to its high abundance in human serum (*ca.* 2-3 mg/mL) and the well-known and widely-studied effect of alcohol on its glycosylation pattern.

2.1 Structure of transferrin

Tf is a protein of *ca.* 79.5 kDa consisting of 679 aminoacids involved in iron transport with two iron binding sites. Asn-413 and Asn-611 carry two potential glycosylation sites which bind to two carbohydrate chains ending with negatively charged sialic acid residues. In normal human serum, glycoforms with two to eight sialic acids have been identified and differ from their p*I*. **Fig. 4.3** presents the structure of the clinically relevant glycoforms and their respective p*I*. Tetrasialo-Tf is the most abundant isoforms, representing *ca.* 75 % of the total glycoforms.

CDT encompasses the glycoforms presenting p*I* ≥ 5.7, *i.e.*, asialo-Tf and disialo-Tf. In 1976, Stibler and Kjellin described an increase in disialo-Tf in serum of alcohol-addicted patients, together with the presence of asialo-Tf, a non sialylated glycoforms which is not observed in normal human condition [47]. The mechanisms inducing Tf glycosylation changes in an alcoholic liver is not yet well understood, but animal and human studies showed a decreased dolichol during glycans synthesis, an inhibition of glycosyltransferases, a reduced β-galactoside α2,6 sialyltranferase gene expression, the

stimulation of cytosolic and plasma membrane sialydases, and the glycans modifications due to the presence of oxidation products of ethanol [2].

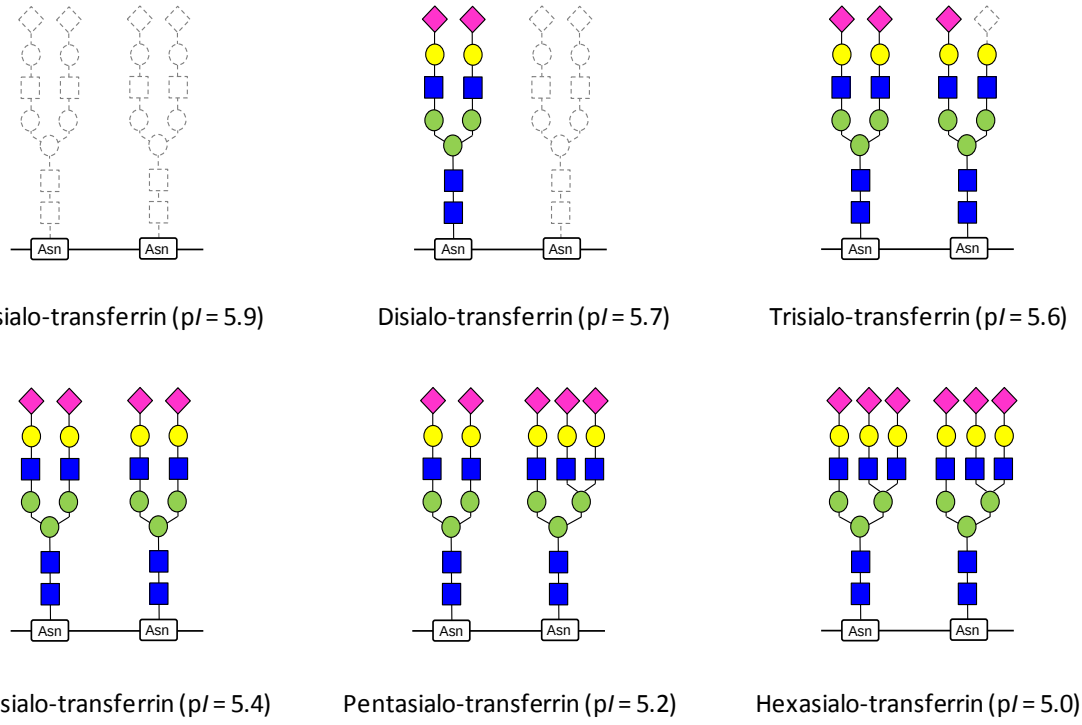

Figure 4.3. Schematic representation of clinically relevant isoforms. Asn, asparagines residue; blue squares, *N*-acetylglucosamine; green circles, mannose; yellow circles, galactose; and purple diamonds, sialic acid. It is worth mentioning that trisialo-Tf contains four glycans chains, composed of one biantennary di-sialylated *N*-glycan and one biantennary mono-sialylated *N*-glycan.

CDT remains today the most widely used biomarker for the diagnosis and the monitoring of chronic alcohol consumption. Depending on the test, its diagnostic specificity is estimated between 89 and 100 %, with a diagnostic sensitivity of 60-70 % [48]. However, its reliability largely depends on the analytical method used for the separation and determination of the glycoforms.

2.2 Analysis

The analysis of CDT is performed in serum. Due to a relatively weak bond between galactose and sialic acid residue, the serum has to be rapidly centrifuged after the collection and stored at 4 °C (up to 7 days) or at -20 °C (several months) [48]. In their first study in 1976, Stibler *et al.* used the isoelectric focusing (IEF) technique to separate the relevant glycoforms. The separation of isoforms by IEF relies on their different electrophoretic mobility in a gradient pH, according to their number of sialic acids and their p*I*s. Due to its high specificity, IEF of serum Tf followed by immunofixation using anti-Tf Ab has been long considered the standard method but suffered from a rather high complexity and analysis time. In the early 1990s, other methods based on column separations followed by immunodetermination of retained and non-retained fractions were proposed and commercialized as kit procedures. However, these methods presented some limitations which led to inaccurate determination of CDT and false-positive or false-negative results, mainly in case of genetic Tf variants. Over the last 15 years, a wide range of analytical strategies were proposed for CDT absolute

or relative quantitation, including HPLC- and CE-based approaches, as well as direct immunoassay determination (immunonephelometric assay). However, until the last decade, neither international standardized procedures nor guidelines were available for clinical and forensic laboratories regarding recommended analytical techniques or cut-off values. This prompted the initiation of a Working Group on Standardization of CDT (WG-CDT) under the auspice of the International Federation of Clinical Chemistry and Laboratory Medicine. The WG-CDT published their first recommendations in 2007 which proposed the following standardization [49]:

(i) Disialo-Tf is defined as the primary target molecule for CDT measurement and represents the single analyte for CDT standardization. Although slightly enhancing the diagnostic specificity, the determination of asialo-Tf has been not considered in the guidelines due to a significant reduction in the diagnostic sensitivity. It is worth mentioning that WG-CDT did not rule out the inclusion of asalio-Tf in CDT, but proposed that CDT *measurement* should use disialo-Tf as the primary (not sole) target molecule [50]. Asialo-Tf determination may be carried out in situations where a high diagnostic specificity is preferred over the sensitivity.

(ii) HPLC is considered as the best candidate for CDT determination due to the relatively specific and sensitive measurement of the iron-Tf complex at λ = 460-470 nm, the adequate separation of glycoforms, the quantification by measuring peak area or peak height, and the easily understood record of peak identification [51]. The proposed HPLC method is based on an anion-exchange chromatographic separation and allows for an almost baseline separation of disialo- and trisialo-Tf.

The WG-CDT group also considered the use of CE less suitable as a reference method due to the rather unspecific UV detection at λ = 200 nm, at which many other biomolecules such as C-reactive protein or complement factors could interfere. Moreover, the analytical sensitivity has been reported to be lower than for HPLC. Nevertheless, despite these statements, CE-UV is largely used in many laboratories for CDT determination [52]. Moreover, nothing is said in the guidelines about a confirmatory analysis. However, as deeply discussed in **Chapter I**, confirmation of presumed positive samples should always occur, mainly in case of legal consequences. In this context, both CE and HPLC methods should be considered for an exhaustive determination.

CE analysis of CDT requires the use of coated capillaries to prevent the protein adsorption and to provide a sufficient glycoforms resolution (mainly for disialo-Tf and trisialo-Tf). The most widely used CE-UV method relies on the use of a bilayer dynamic coating kit which was developed by the group of Lanz and Thormann and is today commercialized by Analis (Suarlée, Belgium) [53-57]. This kit consists of (i) an *initiator* composed of a TRIS/phosphate buffer containing polycations and first flushed within the capillary and (ii) the BGE composed of TRIS/borate buffer at pH 8.5 which contains polyanions. The results obtained for the analysis of an aliquot of serum is presented in **Fig. 4.4**, and highlights the good resolution obtained for the separation of disialo-Tf (P2) and trisialo-Tf (P3).

A recent 10-year retrospective evaluation has shown little variation (lower than 10%) for quality controls and human serum of healthy individual, and a similar robustness of the assay compared to that of HPLC reference method [52]. Nonetheless, as stated by the WG-CDT, the selectivity of this procedure performed with UV detection at λ = 200 nm remains low. Moreover, although the

determination of asialo-Tf is currently not recommended by the standard guidelines, the sensitivity of the method could be improved to provide a better detection of asialo-Tf (P0). CE-MS is thus particularly adapted to enhance both analytical selectivity and sensitivity, as well as provide additional valuable information for medico-legal purpose. Moreover, CE-UV is also prone to co-migration of relevant glycoforms in case of genetic variants. The use of MS detection may help dealing with these samples which are not easily interpretable. MS is also considered as the best technique for a reference method by the WG-CDT.

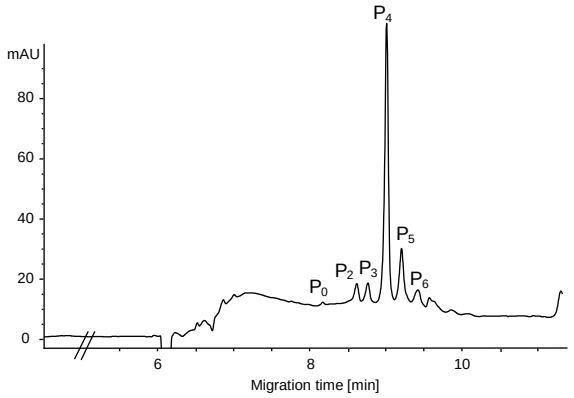

Figure 4.4. Typical electropherogram obtained for a positive sample with the commercial procedure. P0, asialo-Tf; disialo-Tf; P3, trisialo-Tf; P4, tetrasialo-Tf; P5, pentasialo-Tf; and P6, hexasialo-Tf. Experimental conditions are described in **Article IV**.

Due to the presence of the coating agent in the BGE during the separation (*i.e.*, dynamic coating) and the non-volatile buffers, the described procedure is not MS-compatible. In this context, fully compatible CE conditions were developed to ensure a complete method transfer to CE-MS configuration. ESI-MS operating parameters were investigated towards Tf infusion experiments.

3 CE-MS method development

As already discussed in **Section 1**, adsorption phenomenon to the capillary wall is difficult to predict, even more for relatively heavy proteins such as Tf. Preliminary results showed a strong and irreversible adsorption of Tf migrating under its anionic form with a basic BGE. Despite the electrostatic repulsion expected between both negatively charged protein and capillary surface, a substantial adsorption occurred, highlighting the importance of other interactions in the process. Thus, a global methodology was implemented in CE-UV with the screening of numerous coating procedures in combination with different BGE compositions. The combinations were evaluated in terms of peak efficiency, glycoforms resolution, protein mobility, EOF mobility, and baseline detection. Due to the expected interactions between all experimental factors (*e.g.*, BGE composition, pH, nature of the coating, *etc.*), a methodology based on a design of experiments might have been envisaged to reduce the high number of experiments. However, with such experiments involving in-house coating procedures, univariate investigations were carried out due to the high complexity in developing an adapted procedure for each coating (*e.g.*, concentration of the polymer(s), coating procedure, rinsing conditions, regeneration of the coating, *etc.*).

3.1 CE conditions

As deeply discussed in **Article IV**, the methodology involved the screening of anionic, neutral, and cationic coatings consisting of single, double, or triple layers. The selection of the screened coatings was made according to preliminary experiments, a literature survey, as well as the complexity of the in-house coating procedure. For each stable coating, ensured by the analysis of a standard set of low molecular weight compounds, a wide range of were tested, based on volatile electrolytes such as acetic and formic acid, with or without the presence of ammonium counterions, and triethylamine. Numerous acidic and basic BGEs were tested to evaluate the separation performance with positive and negative Tf mobility. **Fig. 4.5** illustrates the electropherograms obtained for each in-house coating procedure with the analysis of a Tf standard solution at 1.5 mg/mL.

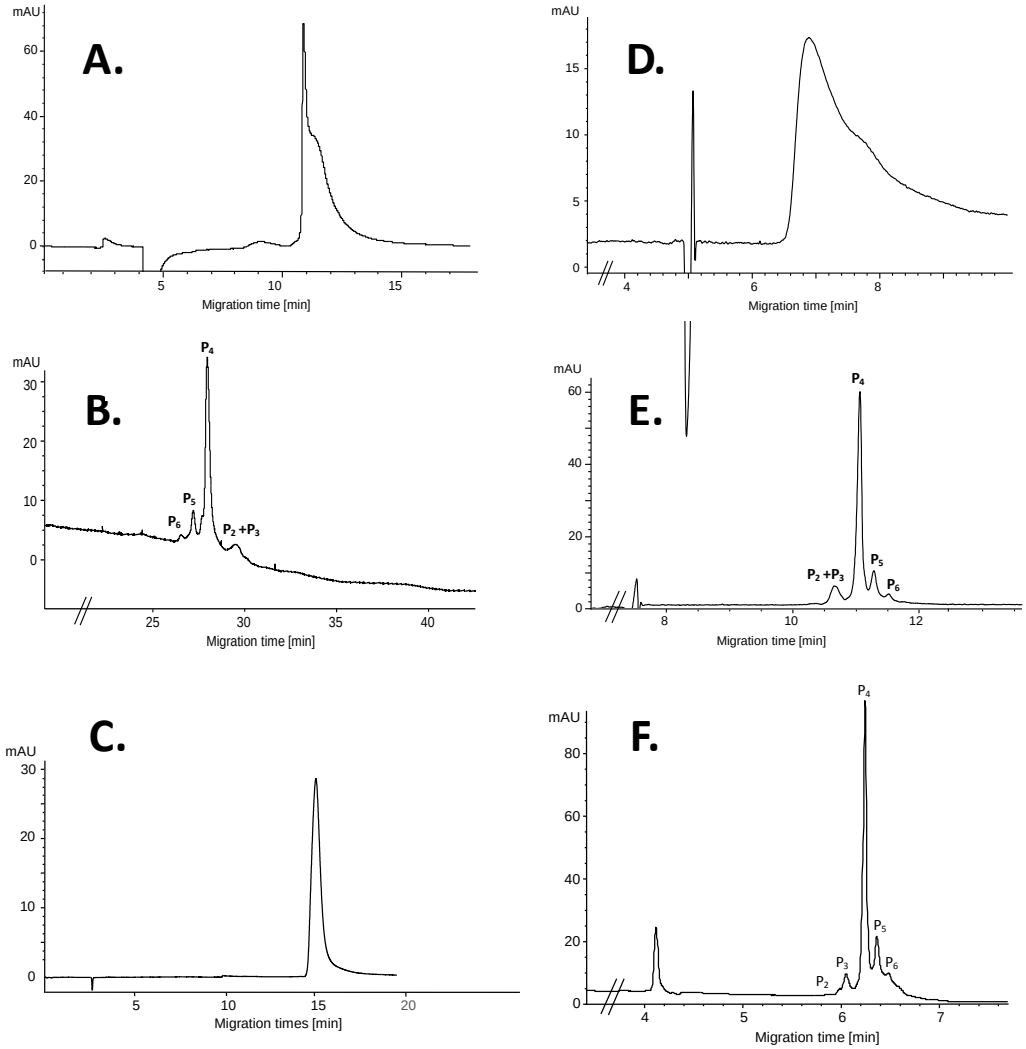

Figure 4.5. Electropherograms obtained in CE-UV with different coatings. A. PEI coating (acidic BGE and negative CE polarity), B. UltraTrol™ LN coating (basic BGE and negative CE polarity), C. UltraTrol™ LN coating (acidic BGE and positive CE polarity), D. CEofix™ MS compatible coating (basic BGE and positive CE polarity), E. PB-PVS coating (basic BGE and positive CE polarity), and F. PB-DS coating (basic BGE and positive CE polarity). All experiments were performed with Tf at 1.5 mg/mL. Other experimental conditions are described in **Article IV**.

According to the selected evaluation criteria, best results were obtained with a PB-DS coating (10 % each, *m/v*) and a BGE composed of 20 mM ammonium acetate at pH 8.5, as illustrated in **Fig. 4.5.F**. Disialo- and trisialo- Tf were not baseline resolved, but the additional selectivity expected with the

MS detection can help discriminate them according to their different *m/z*. Other coatings and conditions either led to poor separation performance or lacked sufficient stability.

The PB-DS coating procedure for new fused-silica capillaries is presented in **Table 4.1**.

Table 4.1. PB-DS coating procedure.

Coating procedure (ΔP = 2 bar)		
Methanol	5 min	(12.8 V_{cap})
Water	3.5 min	(9.0 V_{cap})
1M NaOH	5 min	(12.8 V_{cap})
Water	3.5 min	(9.0 V_{cap})
PB 10%	10 min	(25.7 V_{cap})
Water	3.5 min	(9.0 V_{cap})
DS 10%	10 min	(25.7 V_{cap})
Water	3.5 min	(9.0 V_{cap})
BGE	10 min	(25.7 V_{cap})
Preconditioning (ΔP = 2 bar)		
BGE	3min	(7.7 V_{cap})
Overnight storing		
Capillary filled with BGE and tips placed in BGE vials		

PB-DS coating presented a much higher coating stability compared to the other coatings, especially PB-PVS which also belongs to SMIL coatings, where the DS layer is replaced by a PVS one. The higher stability can be explained by a difference in the thickness of the last layer. A recent study by Haselberg *et al.* used atomic force microscopy to investigate the thickness and surface morphology of multiple SMIL coatings [58]. DS-containing coating has shown to appear *ca.* 1 nm thicker than the corresponding PVS-containing coating, attributed to the molecular structure of the anionic polymer. PVS is a linear polymer, whereas DS is composed of more bulky dextran unit which are prone to branching. Furthermore, significant differences were observed in terms of layer thickness for mono-, bi-, and trilayer coatings. The increase in the layer height was not linear, suggesting that the polymers may occupy a flat or random coil conformation and are spread over the silica surface [58]. **Fig 4.6** offers a schematic representation of the PB-DS coating, highlighting the loop and tail structure encountered by both PB and DS polymers. The apparent surface charge and the conformation of both polyelectrolytes are dependent on their respective concentration and molecular weight [16,17]. **Fig. 4.6.A** schematizes a stable coating, efficient to prevent protein adsorption, *inter alia* by electrostatic repulsions. Once the coating is stable, is it also of utmost importance to keep the coating in appropriate storage conditions (*e.g.,* capillary filled with BGE and tips placed in BGE vial, *cf.* **Table 4.1**) and perform efficient preconditioning steps prior to the analysis to avoid the deterioration of the coating. Otherwise, the DS-loop modifies its structure, which leads to the presence of patches of the PB-loop at the surface of the coating, as illustrated in **Fig. 4.6.B**. PB-layer may then interact with the protein, leading to its unfolding and potential adsorption.

If correctly handled and stored, PB-DS coating has shown to be stable to methanol, acetonitrile, and 1 M NaOH. If necessary, removal of the coating was performed by flushing the capillary with 0.1 M HCl before applying the coating procedure again.

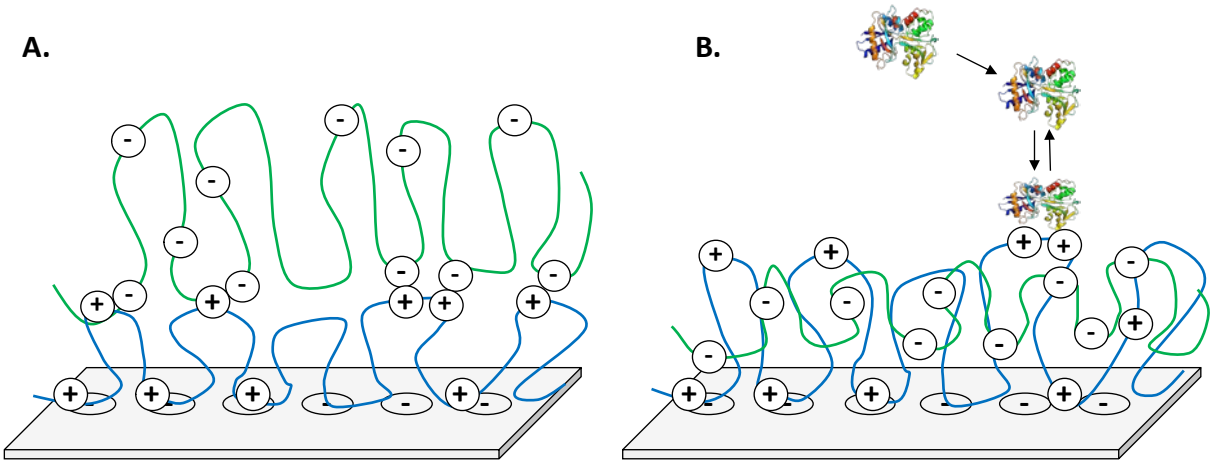

Figure 4.6. Schematic representation of the PB-DS coating. A. Stable coating with appropriate looped structures, B. Effect of inappropriate storage conditions on the coating structure: by modifying the DS-loop, the positive charges of PB-layer become reachable by the protein, leading to protein unfolding and possible adsorption.

3.2 CE-MS method transfer

Over the last decade, the number of publications related to intact protein analysis by CE-ESI-MS has been considerably increasing. Besides the use of model proteins to investigate new separation conditions or interface, relevant applications concerned the characterization of protein structure, the determination of degradation products, as well as glycoforms profiling [11-13]. The latter was involved in studies related to interferon-β [59,60], EPO [61-64], vascular endothelial growth factor [65], or α-1-acid glycoprotein [62,66] determination. These numerous applications highlighted the interest roused by CE-MS for intact (glyco)protein analysis. However, a wide range of proteins remains uncovered by these studies; this is also the case for CDT, where the sole relevant study, published by the group of Sanz-Nebot, concerned its analysis by CE-ESI-TOF/MS and highlighted the numerous challenges encountered due to poor ionization efficiency of the protein [32]. This study was published in 2007 and, at present, no additional successful CE-MS determination of CDT has been found in the literature.

Similar issues in sensitivity were observed in the context of the work presented in **Article IV**, as well as other analytical challenges related to the CE-MS configuration, which are discussed hereafter.

3.2.1 Sensitivity issues

ESI and TOF/MS operating parameters were investigated with infusion experiments, including the composition of the sheath liquid, known as being one of the most crucial parameters to tune the ionization. Numerous experiments were carried out in ESI positive and negative polarity which aimed at producing the highest signal intensities for the protein. Due to the inherent ionization process of ESI source, multicharged ions were detected due to the high charge density of the ESI droplets. With adapted ionization and detection conditions, a multicharged envelop was obtained in positive ionization over the range of 2000 – 3000 *m/z* during Tf infusion experiments, as shown in **Fig. 4.7**. The selected conditions allowed for the detection of the major and more abundant tetrasialo-Tf with a relatively high charge number ($27 \leq z \leq 37$). Among the other relevant glycoforms, only disialo-Tf

could be detected at rather low intensities. The identity of tetrasialo-Tf and disialo-Tf was confirmed by automatic deconvolution of the mass spectrum, as illustrated in **Fig. 4.7.B**.

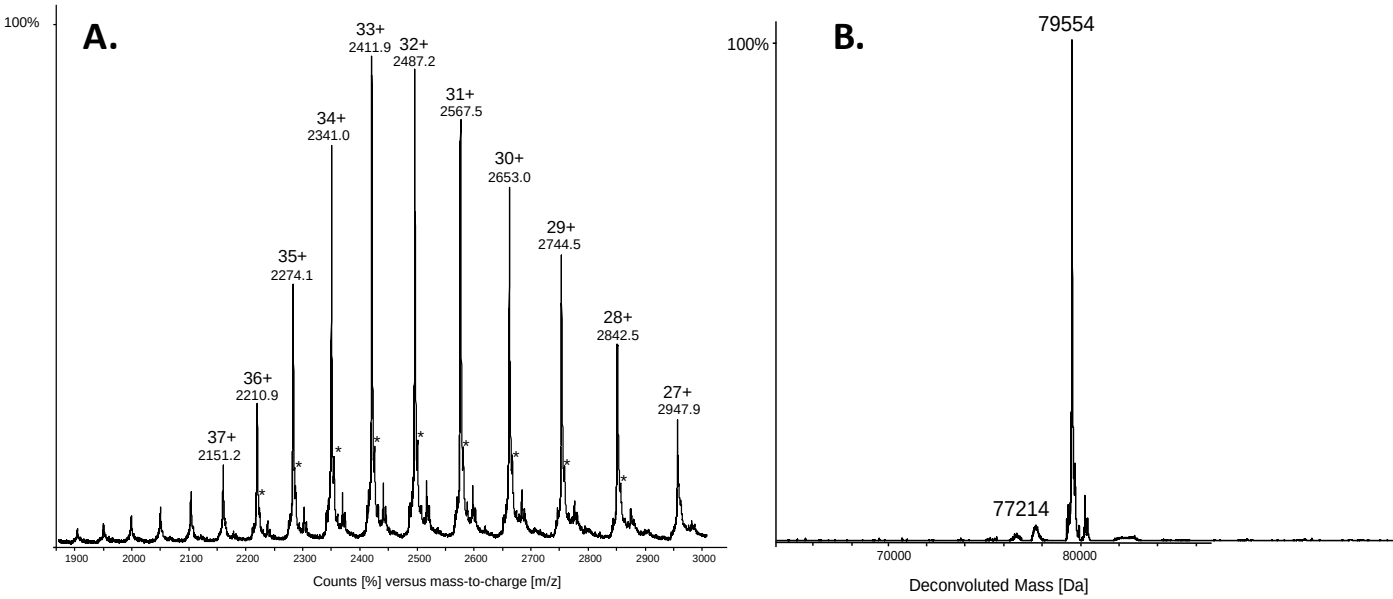

Figure 4.7. Results obtained with ESI-TOF/MS for Tf infusion. A. MS profile observed for tetrasialo-Tf; *stars*: disialo-Tf. B. Deconvoluted mass spectrum.

These infusion experiments brought out two major issues encountered with the detection of Tf, *i.e.*, (i) the poor ionization efficiency, which could not be enhanced whatever the operating parameters and the composition of the sheath liquid, and (ii) the relative complexity of the obtained multicharged spectra. For the latter, it was observed that even in case of disialo-Tf detection, trisialo-Tf was not observed, although the latter should present a higher abundance than the former. This is probably explained by the insufficient resolution of the MS instrument, leading to an overlapping of the multicharged envelops of each glycoform. Indeed, as observed in the mass spectrum presented in **Fig. 4.7.A**, the multicharged envelop of disialo-Tf was almost merged with the one of tetrasialo-Tf. Both glycoforms present a theoretical difference of *ca.* 2200 Da in their molecular mass. Trisialo-Tf, due to the particularity in its structure where only the sialic acid moiety is absent and not the whole glycans chain (*cf.* **Fig. 4.3**) [67], presents a theoretical mass difference of only 273 Da with tetrasialo-Tf. The absence of its detection is thus probably explained by an insufficient mass resolution combined to poor intensities, which makes the automatic or manual mass spectral deconvolution arduous. Furthermore, other multicharged ions were detected in the mass spectrum which did not correspond to any glycoform. Therefore, the protein probably underwent degradation or structural modifications during the analytical process which could lead to deamidation, acetylation, or oxydation products. Nevertheless, due to the low intensities, their identification was not possible.

These sensitivity issues were also confirmed by eventually transferring the whole methodology to CE-ESI-TOF/MS analysis. With a conventional HD injection, no peak was observed nor obtained after the algorithmic protein deconvolution. By increasing the loading quantity, tetrasialo-Tf could be detected, but accompanied with a significant loss in glycoforms resolution.

3.2.2 Resolution issues

The CE-ESI-TOF/MS experiments not only confirmed the issues related to the poor sensitivity, but also highlighted the challenges encountered with the CE-MS transfer. The quality of the glycoforms separation was monitored *via* on-line UV detection, leading to the observation of a modified glycoforms pattern during CE-MS experiments. The modification of this pattern, mainly presenting a loss in glycoforms resolution, could be explained by numerous effects encountered through the whole analysis:

(i) The suction effect caused by the nebulizing gas which induces a hydrodynamic profile within the capillary, deleterious for the separation. However, with the relative great capillary length (129 cm) used during these experiments, this effect is hypothesized to be negligible;
(ii) The variations in the injection process, which may be modified due to the continuous suction effect induced by the nebulizing gas; and
(iii) The moving ion boundary effect, already discussed in **Section 3.2.2.2**, which is encountered due to the migration of the sheath liquid components into the capillary.

Among these hypotheses, the last effect is probably the most significant. Even in presence of a high EOF generated by the PB-DS coating, the small anions of the BGE may be expelled towards the capillary inlet and replaced by anions coming from the sheath liquid. Using different electrolyte compositions for BGE and sheath liquid can increase this phenomenon, which was the case in this context with an acetate-based BGE and a hydro-organic sheath liquid containing formic acid. Furthermore, the effect is expected to be more important in long capillaries and for compounds presenting a negative effective mobility.

3.2.3 Alternatives

Although confirmation of the above-mentioned hypotheses would require further investigations, it seems clear that the sheath-flow configuration is significantly contributing to these issues. Therefore, in the context of CDT determination, the use of the sheathless interface should be considered. First, the overall sensitivity is expected to be enhanced with absence of the dilution observed in the sheath-flow configuration. Secondly, by allowing for a direct ionization of the CE effluent without liquid addition or pneumatic assistance, neither the suction nor the moving ion boundary effects might occur. Furthermore, the protein conformation is expected to remain similar during the separation until the droplets evaporation, with an ionization process mostly depending on the BGE composition. The sheath liquid is usually considered an attractive parameter to tune the ionization conditions. However, the high organic content can lead to changes of the protein conformation, which can also be modified at very low or very high pH, often encountered with the addition of strong acid or alkali in the sheath liquid. The sheathless interface based on Moini's prototype has recently shown acceptable performance, especially in terms of sensitivity, for the determination of the glycoforms pattern of pharmaceutical proteins by using neutral coated capillaries and acidic BGE conditions [59].

Currently, only bare-fused silica, PEI, and neutral coated capillaries are manufactured by Beckman Coulter for the CESI interface and are not commercially available. In this context, in order to evaluate the potential of sheathless configuration in the determination of CDT, fused silica capillaries were tentatively coated by applying a slightly modified PB-DS procedure. However, the high viscosity of the 10 % solutions was not adapted to the etched 30-µm i.d. capillaries. The capillary was immediately clogged after the procedure and the etched tip damaged. Therefore, further investigations in the coating procedure and the polymer concentrations are required to be fully adapted to the smaller i.d. and the tenuous tip. The use of the manufactured neutral coated capillaries could also be envisaged, due to the probable similar polyacrylamide composition compared to that of UltraTrol™ LN coating which was used in this study and gave relatively acceptable results in terms of glycoforms resolution.

3.3 Introducing Article IV

The study presented in **Article IV** consisted in two sequential sections, *i.e.*, the development of MS-compatible conditions in CE-UV configuration for the determination of CDT glycoforms, followed by CE-ESI-TOF/MS method transfer. The investigations of MS-compatible conditions included the screening of numerous coating compositions and procedures, as well as BGEs. In order to increase the probability of having an acceptable glycoforms separation, numerous anionic, neutral, and cationic coatings were investigated. Some coatings were unstable, even with a careful investigation of the procedure, while others gave separation of bad quality. PB-DS showed the best compromise for coating stability and CDT determination (*e.g.,* resolution, efficiency, migration times' repeatability, *etc.*). All coating procedures were in-house developed by evaluating various polymer concentrations and flushing steps (*e.g.*, pressure, duration, waiting time, *etc.*).

The second part of the study consisted of the evaluation of the ESI-MS parameters, including the nebulizing gas flow rate, the drying gas flow rate and temperature, the ESI and fragmentor voltages, and the sheath liquid composition. In order to increase the ion transmission efficiency, MS octopole and detector parameters were also tuned. For the latter, increasing the voltages applied to the microchannel plate and the photomultiplier tube led to higher signal intensities, but also higher background noise which hindered the algorithmic deconvolution of the multicharged envelop. Due to the low sensitivity still encountered with the CE-ESI-TOF/MS configuration even with adapted parameters, the same infusion experiments were carried out with an Agilent 6530 Accurate-Mass Q-TOF LC/MS System which presents improved sensitivity and mass resolution compared to the Agilent 6210 TOF LC/MS System. The QTOF instrument is also equipped with the Jet Stream source, thus requiring careful optimization of the sheath gas parameters. However, the gain in sensitivity obtained with the QTOF was not significant.

Article IV also raised the need for further studies regarding the effect of the sheath-flow configuration in the quality of the separation. Although these inherent effects are most frequently not deleterious for a separation (and even not remarked), they seemed to become significant in complex analytical challenges, such as those encountered with glycoform profiling. Complementary systematic investigations are thus required for a better understanding of this phenomenon.

4 Conclusions

This study highlighted the numerous issues that can be involved in intact glycoprotein analysis by CE-MS. First, a strategy to prevent the adsorption of proteins to the silica surface has most frequently to be implemented. Capillary coatings have been widely used for this purpose. Dynamic coatings provide satisfactory performance but cannot be used with MS detection due to their continuous presence in the BGE. A careful selection of the nature of the coating is also mandatory due to the low volatility of some polymers. Moreover, the development of a coating procedure remains tedious and time-consuming. Secondly, high separation efficiency is required to provide a sufficient resolution between the glycoforms. Due to the MS detection, only volatile electrolytes can be selected which narrows the possible BGE compositions. It has been shown by Eriksson *et al.* that the use of some non-volatile electrolytes such as borate (on the contrary of phosphate) could be envisaged for the determination of small proteins without significant ion suppression. However, this strategy seemed to be not adapted to the analysis of complex proteins such as glycoproteins, leading to troublesome ionization [68]. Then, even if some glycoproteins, *e.g.*, EPO, show acceptable ionization behavior as reported in the literature a vast majority of complex (glyco)proteins are not easily ionized which, in combination with the low volume injected, leads to poor sensitivities. This is particularly true for large and heavy proteins (≥ 30 kDa) such as Tf or monoclonal Abs.

In the context of CDT analysis, this study also brought out the complexity of the sheath-flow interface and its effect on the quality of the separation. Beside the limitations already encountered when using MS-compatible conditions, the direct transfer of MS-compatible CE-UV method to CE-ESI-MS configuration led to unsatisfying performance due to the deleterious effect of the interface onto the separation. A more appropriate strategy might be to directly investigate the parameters in CE-MS configuration. However, the use of in-house coated capillaries impedes this approach due to the complexity of developing a coating procedure. It is worth mentioning that covalently coated capillaries are commercially available; however, their prices often remain prohibitive (especially in method development when too harsh conditions can be used) and they are not necessarily adapted to the analytical requirement.

Not only the separation, ionization, and detection of (glyco)proteins are important, but also the sample purification. Protein characterization of biopharmaceuticals often involves a very simple sample pre-treatment due to the low number of sample constituents (*e.g.*, excipients, degradation products, *etc.*). However, in case of biological matrices, the preparation is much more complex. Due to its serum abundance (mg/mL range), Tf presents an advantage over the other proteins often found at very low concentrations in blood. Nevertheless, the presence of albumin and IgG in the sample is deleterious for the quality of the separation (potential additional adsorption, co-migration, signal suppression in MS). Immunodepletion kits can be used to remove these high abundant proteins; however, numerous commercial kits (*e.g.*, ProteoPrep© 20 Plasma Immunodepletion Kit, Sigma-Aldrich) are developed for proteomics applications and, thus, also involve the depletion of Tf. Sigma-Aldrich also proposes a kit the depletion of albumin and IgG but removing these both proteins should not be enough for Tf analysis in the developed operating conditions. Selective protein extraction should be thus envisaged to obtain a sufficient clean-up. Until few years ago, a commercial kit based on spin columns and Ab-Ag recognition with avian IgY Abs could be purchased

(ProteomeLab™ IgY Transferrin Spin Column Proteome Partitioning Kit, Beckman Coulter) but is not commercially available anymore. An analogous procedure has been recently described by Mesbah *et al.* with the in-house spin column preparation followed by immunoextraction [69]. In the context of this work, the procedure was applied to the extraction of Tf prior to the CE-UV analysis with the developed PB-DS-based conditions. Preliminary experiments showed the relative complexity of the entire procedure, *i.e.*, coupling of Ab to the spin column, immuno-capture of Tf, rinsing procedures, and stripping of Tf, spin column regeneration, which is time-consuming, requiring a full-time activity during more than one day. Furthermore, the procedure was developed for the determination of CDT with the commercial analysis kit and is not completely adapted to the PB-DS conditions. As an example, bovine serum albumin is added at this end of the procedure to facilitate the refolding of Tf in a medium close to a serum protein concentration. The addition of albumin was found deleterious in the developed separation conditions with a probable adsorption to the coating. Therefore, the procedure should be further investigated to allow its integration within the developed method.

Finally, it is worth mentioning that CE-MS may also be successfully used in the determination of alternative indirect or direct biomarkers of alcohol consumption and has already shown its applicability for EtG, ethyl sulfate, or phophatidylethanol analysis [70-72].

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6 Scientific publication

Article IV

New insight in carbohydrate-deficient transferrin analysis with capillary electrophoresis - mass spectrometry

I. Kohler, M. Augsburger, S. Rudaz, J. Schappler, submitted to Forensic Science International.

New insight in carbohydrate-deficient transferrin analysis with capillary electrophoresis – mass spectrometry

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Abstract

Capillary electrophoresis (CE) with UV detection has been widely used for the determination of carbohydrate-deficient transferrin (CDT), an indirect marker of the chronic alcohol consumption (≥ 60-80 g/day). A commercially available method (CEofix™ CDT kit), containing a bilayer anionic coating, allows for the analysis of CDT with a high resolution between transferrin (Tf) glycoforms without protein adsorption onto the capillary wall. Although widely used in routine analysis, this procedure presents some limitations in terms of selectivity and sensitivity which may be overcome with mass spectrometry (MS). However, the available method is not MS-compatible due to the non-volatile coating as well as the phosphate and borate buffers present in the background electrolyte (BGE). This study firstly consisted in developing MS-compatible separation conditions, *i.e.*, coating and BGE compositions. Numerous cationic, neutral, and anionic coatings were evaluated in combination with BGEs covering a broad range of pH values. An anionic PB-DS coating (10% each) combined with a BGE composed of 20 mM ammonium acetate at pH 8.5 provided the best results in terms of glycoforms' resolution, efficiency, adsorption reduction, migration times' repeatability, and coating stability. The method was then transferred to CE-MS after investigations of the electrospray ionization (ESI) source, equipped with a sheath-flow interface, and the time-of-flight (TOF/MS) parameters. A successful MS detection of tetrasialo-Tf was obtained during infusion, while the experiments highlighted the challenges and issues encountered with intact glycoprotein analysis by CE-ESI-MS.

Keywords

Capillary electrophoresis, mass spectrometry, carbohydrate-deficient transferrin, chronic alcohol consumption, capillary coating

1 Introduction

Alcohol misuse or dependence is nowadays of major concern due to the negative impact of ethanol on physiological and psychological condition with health and social issues. The diagnosis of chronic alcoholism (≥ 60-80 g of ethanol/day over a long time) is therefore very important in clinical and forensic toxicology (*e.g.*, occupational medicine, drunk driving, license reapplication, etc.) [1,2]. Two kinds of biomarkers related to alcohol consumption have been considered for the diagnosis of its abuse, *i.e.*, (i) direct markers, such as ethyl glucuronide (EtG), ethyl sulfate (EtS), phosphatidylethanol (PEth), and fatty acid ethyl esters, which derive from alcohol metabolism, and (ii) indirect markers, such as aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), γ-glutamyltransferase (GGT), erythrocyte mean corpuscular volume (MCV), and carbohydrate-deficient transferrin (CDT), whose levels reflect the toxic effect of chronic use of ethanol on tissues and biochemical pathways [3]. The determination of these biomarkers by immunoassays or separation techniques differs regarding their specificity and sensitivity. CDT is currently considered the most relevant biomarker due to its high specificity and, with a mean-life of *ca.* 14-17 days, is also responsive to short-term reductions or increases in alcohol consumption [4].

Human transferrin (Tf) is a serum glycoprotein of *ca.* 79.5 kDa consisting in 679 aminoacids and is involved in iron transport with the presence of two iron-binding sites. Asn-413 and Asn-611 carry two potential glycosylation sites which usually bind two complex *N*-linked carbohydrate chains composed of *N*-acetylglucosamine, mannose, galactose, and ending with negatively charged sialic acid residues. Glycoforms with two to eight sialic acids have been identified in normal human serum and differ in their isoelectric point (p*I*) after complete iron saturation. Tetrasialo-Tf, which consists in two biantennary *N*-glycans with a total number of four sialic acids, is the most abundant isoform (*ca.* 75% of total glycoforms) [5]. Tetrasialo-Tf (p*I* 5.4) and clinically relevant isoforms including two (disialo-Tf, p*I* 5.7), three (trisialo-Tf, p*I* 5.6), five (pentasialo-Tf, p*I* 5.2), and six (hexasialo-Tf, *pI* 5.0) sialic acid residues are presented in **Fig. 1**.

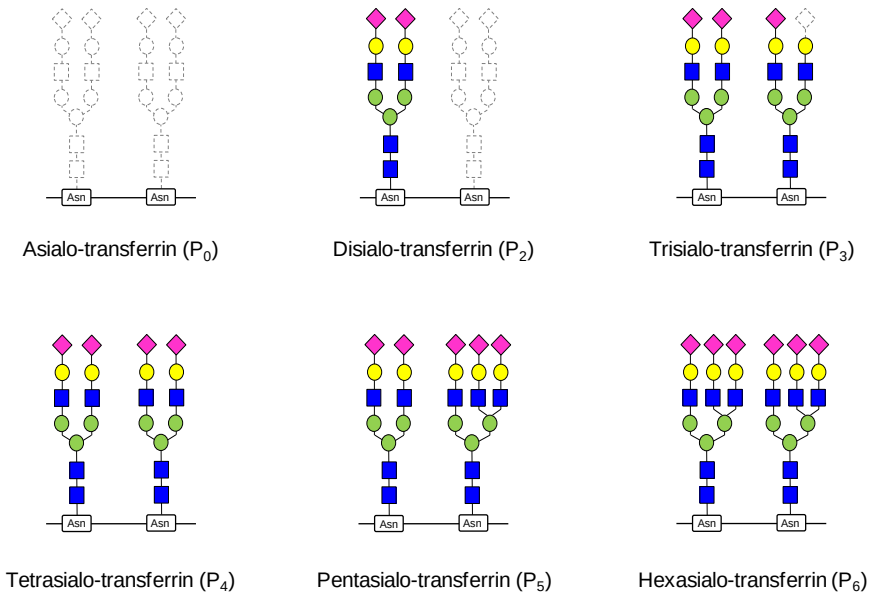

Figure 1. Schematic representation of clinically relevant Tf isoforms. Asn, asparagine residue; blue squares, *N*-acetylglucosamine; green circles, mannose; yellow circles, galactose; and purple diamonds, sialic acid. Trisialo-Tf (P3) contains one biantennary di-sialylated *N*-glycan and one biantennary mono-sialylated *N*-glycan [44].

In 1976, using isoelectric focusing technique [4], Stibler and Kjellin described an increase in disialo-Tf in serum and cerebrospinal fluid of alcohol-addicted patients, together with the presence of a non-sialylated Tf glycoform (asialo-Tf, p*I* 5.9). Isoforms with p*I* ≥ 5.7, *i.e.*, asialo- and disialo-Tf, were later termed CDT. The modification of the isoforms' repartition is likely explained by an influence of ethanol and/or metabolites during *N*-glycan chain synthesis in the Golgi apparatus, which is influenced not only for Tf but also for other proteins [6].

The recommended strategy for the assessment of CDT is based on a two-step workflow which starts with a screening analysis by immunoassays, multi-capillary electrophoresis, or high performance liquid chromatography (HPLC). The screening step is followed by a confirmatory analysis based on capillary electrophoresis with UV detection (CE-UV) or HPLC which allows for the glycoforms patterns visualization and confirmation [7-9]. The Working Group on CDT Standardization of the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC-WG-CDT) recently recommended the use of standardized HPLC-UV at 470 nm (iron-Tf complex) for the determination of CDT. Moreover, disialo-Tf should be the primary target molecule for CDT determination, expressed in a relative amount to total Tf (% CDT). The determination of asialo-Tf slightly improves the diagnostic specificity due to its absence in abstinent and socially drinking subjects. Therefore, CDT is routinely most often measured as the relative sum of asialo-Tf and disialo-Tf to total Tf [2,8,10,11].

CE provides numerous advantages such as short analytical time, high resolution, as well as low solvent and sample consumption. Intact glycoprotein analysis by CE leads to a high efficiency due to the inverse relation proportion between molecular diffusion term and efficiency. Large molecules having low diffusion coefficients, they exhibit little zone broadening [12]. Moreover, a high selectivity between proteins or isoforms can be observed due to large differences in their charge-to-size ratios and hydrophobicity [13]. However, the main drawback is the potential adsorption of biomolecules to the negatively charged capillary wall, due to electrostatic and/or hydrogen bonds interactions. The protein adsorption can be reversible, leading to alteration of peak efficiency and protein mobility, or irreversible, with modification in protein recovery and electro-osmotic flow (EOF) mobility. Numerous strategies have been developed to reduce the adsorption, the most commonly used being to coat the capillary with covalently linked or physically adsorbed polymers [14-17]. The application of these shielding polymers aims at (i) sterically masking residual silanol groups to reduce electrostatic interactions, and (ii) increasing the viscosity at the boundary of the capillary wall to hinder the accessibility to the proteins [16].

Over the last decade, some developments with coating approaches have been made to prevent Tf adsorption onto the capillary wall and allow for the glycoforms discrimination. Based on these developments, a commercial procedure (CEofix^{TM} CDT kit, Analis, Suarlée, Belgium) has been proposed for CDT determination, including reagents for iron saturation prior to the injection, dynamic bilayer coating of the capillary wall, and TRIS borate buffer background electrolyte (BGE) [18-22]. Although being nowadays widely used for routine analysis with adequate performance [11], this method suffers from two main drawbacks: (i) a rather low selectivity due to the UV detection at 200 nm, at which many other biomolecules with close p*I* (*e.g.*, C-reactive protein, complement factors, etc.) can interfere [8], and (ii) a relatively poor sensitivity due to the very low quantity injected and the short optical pathway provided by the internal diameter of the capillary [23]. Both

selectivity and sensitivity can be improved with the use of mass spectrometry (MS) but the procedure is not MS-compatible due to non-volatile buffers and dynamic capillary coating.

This study consisted in developing MS-compatible conditions for the determination of CDT by CE-MS with a time-of-flight (TOF) mass analyzer. Numerous capillary coatings, as well as BGE composition, were screened and evaluated in terms of adsorption prevention, glycoforms resolution, efficiency, changes in baseline, and migration times' repeatability. Electrospray ionization source (ESI) equipped with a sheath-liquid interface [24] and TOF/MS parameters were also investigated towards Tf ionization and detection.

2 Materials and methods

2.1 Chemicals

Methanol (MeOH), isopropanol (*i*-PrOH), acetonitrile (MeCN), glacial acetic acetid, formic acid, and ammonium acetate were purchased from Biosolve (Valkenswaard, The Netherlands) and were all ULC/MS grade. Ammonium formate, hydrochloric acid, and sodium hydroxide were obtained from Fluka (Buchs, Switzerland). 28% ammonia solution (*m/v*), acetone, triethylamine (TEA), hexadimethrine bromide (Polybrene, PB, M_w ~15,000), poly(vinylsulfonic acid, sodium salt) (PVS) water solution (25%, *m/v*), dextran sulfate (DS) sodium salt (M_w ~500,000), and human Tf were purchased from Sigma-Aldrich (Seelze, Germany). Trimethoxysilylpropyl(polyethyleneimine) (PEI), 50% in *i*-PrOH (*m/v*), was obtained from Gelest, Inc (Morrisville, PA, USA). UltraTrol™ Dynamic Pre-Coating LN was purchased from Target Discovery (Palo Alto, CA, USA). CEofix™ CDT kit and CEofix™ MS kit were obtained from Analis (Suarlée, Belgium). Ultrapure water was supplied by a Milli-Q Advantage A10 purification system from Millipore (Bedford, MA, USA).

2.2 Coating procedures and BGE

2.2.1 Capillary coatings

For each coating, new capillaries (BGB Analytik AG, Böckten, Switzerland) were first flushed at 2 bar with MeOH (5 min), water (5 min), 0.1 M HCl (5 min), and water (5 min) prior to the coating procedure. The latter involved capillary rinses with polymeric aqueous solutions to form a physically adsorbed mono-, bi- or trilayer coating. The prepared polymeric aqueous solutions were filtered prior to the coating procedure through a 0.45-µm nylon filter (BGB Analytik AG, Böckten, Switzerland). Each procedure is described in Supplementary data **Table 1**, classified in anionic, cationic or neutral coating, depending on the polymeric composition of the top layer.

2.2.2 BGE composition

Numerous BGEs were investigated for each coating procedure, *i.e.*, (i) acetic and formic acid (0.5 – 2 M), (ii) ammonium acetate or formate (10 – 50 mM), prepared at a fixed concentration of ammonium salt with the pH adjusted with ammonium hydroxide, and (iii) TEA (10 – 25 mM) at fixed concentration with the pH adjusted with acetic acid, when necessary. Depending on the charge of the coating top layer (*i.e.*, anionic, cationic, or neutral coating), the pH of the BGE was tested

between 2 and 3, and from 7.5 to 11.7. All investigated BGEs are listed in Supplementary data **Table 2**. For optimized PB-DS coating, the BGE consisted in 20 mM ammonium acetate at pH 8.5. The pH values were measured with a Seven Multi S40 pH-meter from MettlerToledo (Greifensee, Switzerland).

2.3 Samples

The method development was carried out with an aqueous standard solution of Tf at 1.5 mg/mL. Positive plasma samples obtained from the University Center of Legal Medicine (Lausanne, Switzerland) stored at -20 °C were defrosted at ambient temperature and injected after dilution with the Fe^{3+} solution included in the CEofix™ CDT kit.

2.4 Instrumentation

2.4.1 CE-UV

CE-UV experiments were performed with a G7100 CE system from Agilent Technologies (Waldbronn, Germany), equipped with an on-capillary diode array detector, an autosampler and a power supply able to deliver up to 30 kV. Separations were performed at 25°C using a fused-silica capillary with a total length of 64.5 cm, an effective length of 56 cm, and an internal diameter of 50 µm. Hydrodynamic (HD) injection was performed at 30 mbar for 5 s (corresponding to 0.32% of the capillary length, 4 nL injected) followed by a post-plug of BGE at 50 mbar for 2 s. For cationic coatings, experiments were performed in the negative polarity mode (anode at the outlet) with a constant voltage of -30 kV (initial ramping of -1667 V/s), while experiments with anionic coatings were performed in positive polarity mode (cathode at the outlet) with a constant voltage of 30 kV (initial ramping of 1667 V/s). Both polarities were tested for the neutral coating UltraTrol™ LN. UV/Vis detection of Tf was carried out at 195 nm, both with a reference wavelength at 450 nm. EOF mobility (μ_{EOF}) was determined with the analysis of 5% acetone in water (*v/v*) monitored at 260 nm.

2.4.2 CE-MS

CE-MS experiments were performed with a G7100 CE system from Agilent Technologies (Waldbronn, Germany), equipped with an on-capillary diode array detector, an autosampler and a power supply able to deliver up to 30 kV. Separations were performed with PB-DS coated capillaries (see supplementary data **Table 1**) with a total length of 129 cm with UV detection at 56 cm, and an internal diameter of 50 µm. HD injection was performed at numerous pressure values and injection times, followed by a post-plug of BGE at 50 mbar for 5 s. CE was coupled to a 6210 LC/MS TOF mass spectrometer from Agilent Technologies (Santa Clara, CA, USA) via a coaxial sheath-flow electrospray ionisation (ESI) interface from Agilent Technologies (Waldbronn, Germany). The sheath liquid was composed of *i*-PrOH–water (50:50, *v/v*) with 5% formic acid and was delivered at a flow rate of 4 µL/min. Drying gas flow rate and temperature were set at 4 L/min and 350°C, respectively. Nebulizing gas was set at 4 psi. The applied ESI voltage was set at +4500 V. Fragmentor, skimmer, and first octopole voltages were set at 400 V, 65 V, and 250 V, respectively. MS detection was carried out between 1000 and 3200 *m/z*, and 1 spectrum/s was acquired (1000 ms/spectrum, 9528 transients/spectrum). CE was also coupled to a 6530 Accurate-Mass qTOF LC/MS from Agilent

Technologies equipped with an Agilent Jet Stream coaxial sheath-flow ESI source from Agilent. Drying gas flow rate and temperature were set at 16 L/min and 200 °C, respectively. Nebulizing gas was set at 8 psi. Sheath gas flow rate and temperature were fixed at 3.5 L/min and 200 °C, respectively. The applied ESI and nozzle voltages were both set at +2000 V.

2.4.3 Software

CE ChemStation version B.04.02 was used for CE control and MassHunter version B.02.00 (both Agilent Technologies) was used for ESI-TOF/MS control, data acquisition, data handling, and spectral deconvolution.

3 Results and discussion

3.1 Method development

3.1.1 Capillary coating

Whether polymers are present in the BGE or not, physically adsorbed coatings are considered as dynamic or static, respectively. The procedure used in routine analysis for CDT determination is a dynamic coating generated by the addition of water-soluble polymers into the BGE which are continuously present during the separation. The first step of the routine procedure consists of an initial rinse of the capillary with the polycationic buffer solution ("initiator") followed by a flush of the borate separation buffer which contains polyanions ("buffer"). The separation is then carried out and **Fig. 2.A** shows the typical electropherogram obtained for a positive serum sample. This procedure leads to an acceptable resolution between the glycoforms (Rs ≥ 1.7), allowing for the relative quantitation of CDT. Dynamic coatings are simple and show a high stability; however, due to the constant presence of non-volatile polymers within the BGE, they are not MS-compatible. Static-adsorbed coatings are made by flushing the capillary with one or more polymeric solutions prior to the injection. These coatings are semi-permanent and the presence of the polymers in the BGE during the separation is not necessary. They can be used for CE-MS analysis, providing (i) the coating procedure is performed with the capillary outlet tip out of the ESI source and (ii) no bleeding of the coating is observed during the analysis [14,15].

Even in the case of known p*I*, the protein adsorption to the capillary wall or coated polymers is relatively difficult to anticipate due to the multiple interactions that can be involved in the adsorption process (electrostatic interactions, hydrogen bond, dipole-dipole, or dispersion) and the heterogeneous charge distribution on the protein surface, particularly for heavy proteins. Moreover, the adsorption is influenced by the composition and pH of the BGE, the addition of organic solvent, and the temperature [16]. In this study, numerous mono-, bi-, or trilayer static coatings were thus considered and investigated in terms of polymer concentrations, coating procedure, capillary rinses, and regeneration between runs. The coating stability was first ensured with the analysis of a standard set of low-molecular weight compounds (*i.e.*, mixtures of cocaine, methadone, and MDMA at 50 µg/mL, and salicylic acid, ibuprofene, and warfarine at 50 µg/mL). The coating was considered stable with migration times RSDs ≤ 3 %. The analysis of Tf standard (1.5 mg/mL) was then performed

over a wide range of pH with a BGE composed of acetic acid (pH ≤ 2.6) and 20 mM ammonium acetate (pH ≥ 7.5), allowing for a positive and negative effective mobility (μ_{eff}) of the protein, respectively. Coatings were evaluated according to different criteria to estimate the reduction of protein adsorption. Efficiency and protein mobility were measured to monitor reversible adsorption, while EOF mobility and changes in the baseline were monitored to estimate irreversible adsorption [15]. Glycoforms resolution and coating stability were assessed to estimate the performance of the procedures for Tf isoforms analysis. **Fig. 2** presents the electropherograms obtained for each coating that led to Tf detection.

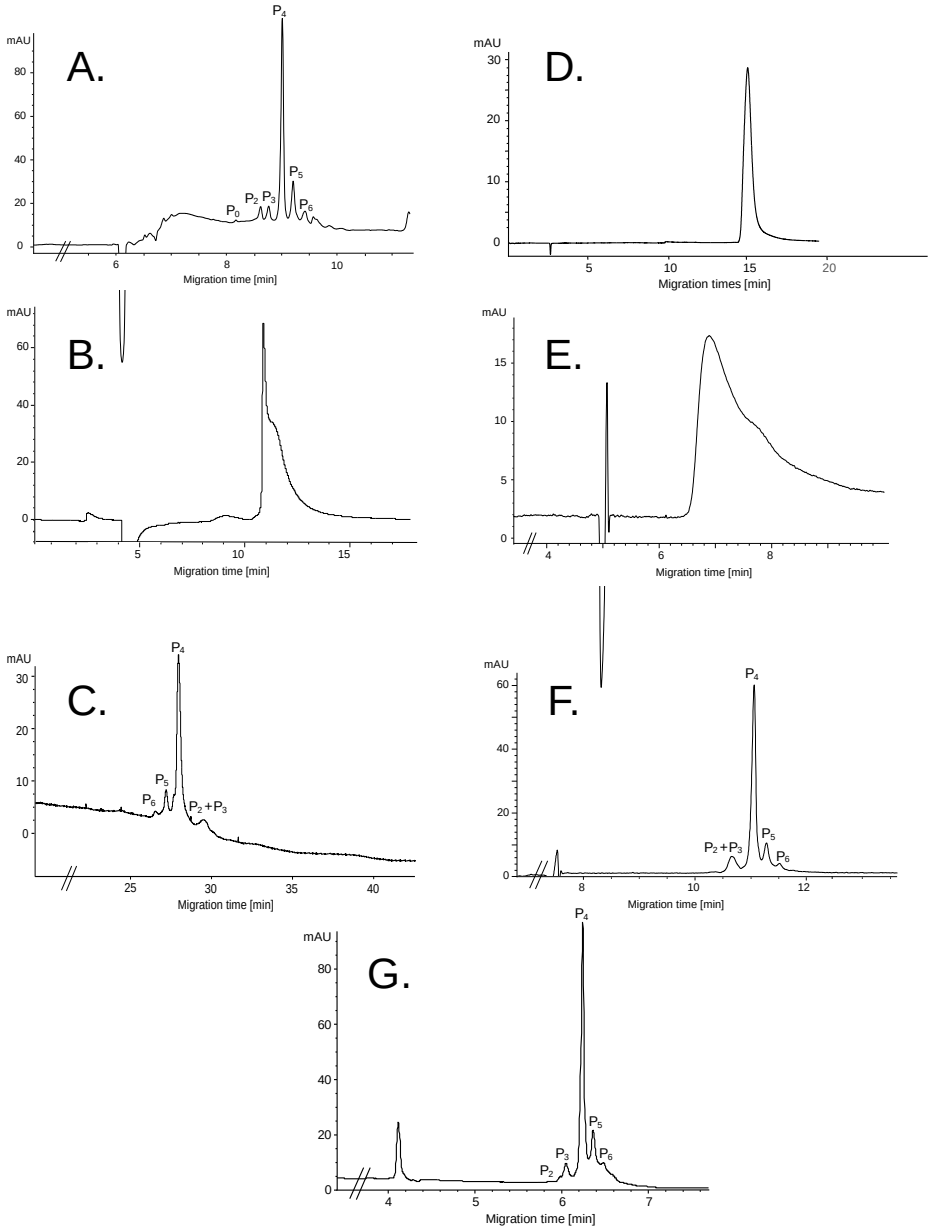

Figure 2. Electropherograms obtained in CE-UV with the different coating procedures. All electropherograms, except in A, were obtained with Tf at 1.5 mg/mL. A. positive serum sample analyzed with CEofix™ CDT kit, B. PEI (10%, *m/v*) coating with an acidic BGE and -30 kV, C. UltraTrol™ LN coating with a basic BGE and -30 kV, D. UltraTrol™ LN coating with an acidic BGE and +30 kV, E. CEofix™ MS compatible coating with a basic BGE and +30 kV, F. PB-PVS (10%, *m/v*, and 5%, *v/v*, respectively) with a basic BGE and +30 kV, G. PB-DS (10% each, *m/v*) coating with a basic BGE and +30 kV. See Sections 2.2 and 2.4.1 for experimental conditions.

3.1.1.1 Cationic coatings

After silanols deprotonation by NaOH rinse, cationic coatings were obtained with (i) a single flush of a polycationic solution (*e.g.*, PEI coating), or (ii) a successive multiple ionic-polymer layer (SMIL) strategy, *i.e.*, successive additional flushes with polyanions and polycations, ending with a polycationic layer, usually forming a trilayer coating (*e.g.*, PB-DS-PB) [25].

The use of a PEI (10%, *m/v*) coating combined with an acidic BGE (2 M acetic acid, pH 2.2) and a reverse polarity led to the migration of Tf as a globally positively charged species. Although an electrostatic repulsion between the positively charged protein and the cationic coating was expected, a significant peak tailing was observed as a characteristic profile of protein adsorption [26], leading to a poor resolution between the glycoforms as illustrated in **Fig. 2.B.**

The trilayer and stable PB-DS-PB coating has been successfully used for the analysis of model proteins and some biopharmaceuticals [27-29]. However, this coating was not adapted to the analysis of Tf. Whatever the polymer concentration, the coating procedure, and the BGE composition and pH, Tf was not detected, possibly due to a strong and irreversible adsorption to the coating.

Therefore, the investigated cationic coatings did not show acceptable results with Tf adsorption to the coating polymers and were discarded.

3.1.1.2 Neutral coating

Neutral coatings, obtained with the attachment of neutral polymers to the capillary wall, reduce the protein absorption and decrease the EOF value (μ_{EOF} ~0). UltraTrol™ LN is a commercial linear polyacrylamide, N-substituted acrylamide copolymer used in CE-MS to prevent protein adsorption and lower the EOF, with the possibility to be completely stripped from the surface capillary with a 1 M NaOH rinse. These conditions have been already successfully applied to the separation of Tf glycoforms in CE-UV with 32.5 mM sodium borate buffer BGE at pH 8.3 [30]. The electropherogram obtained with 20 mM ammonium acetate BGE at pH 8.5 and a reverse polarity showed an acceptable glycoforms separation (R_s ≥1.8), except between disialo- and trisialo-Tf (**Fig. 2.C**). Protein adsorption was prevented and RSD lower than 0.2% was obtained for the migration times. Regeneration of the coating was mandatory after each analysis and performed with a flush of the coating solution. Nevertheless, the efficiencies were relatively low (N ≤ 30,000) with the acetate-based BGE, also decreasing the overall sensitivity, and migration time was rather long (> 25 min). Acidic conditions were also tested with a 2 M acetic acid BGE and a cathodic detection of the protein, as shown in **Fig. 2.D**. Although the protein adsorption was reduced, a single peak was detected without any resolution between the glycoforms.

Despite acceptable results with this neutral coating and a reverse polarity, very low EOF was observed, which can induce unstable CE-MS analyses due to pH shifts inside the capillary with counterions coming from the sheath liquid that can enter the capillary. Air can also be sucked in the capillary, leading to unstable CE currents [31,32]. These issues can be hindered with anionic coatings which present a high and repeatable EOF towards the ESI source (normal CE polarity mode), leading to much stable CE-MS analyses.

3.1.1.3 Anionic coatings

Numerous anionic coatings have been broadly used for protein analysis by CE-MS, *e.g.*, PB-PVS [29,33-35], CEofix™ MS kit [33] or PB-DS [36]. The commercially available bilayer CEofix™ MS-compatible method was predominantly used for the analysis of low-molecular weight compounds [37,38], but can be also envisaged for the analysis of intact proteins [33]. Nevertheless, as highlighted by the electropherogram shown in **Fig. 2.E**, this coating was clearly not adapted to the separation of Tf glycoforms in basic conditions due to a strong peak tailing, indicating a significant protein adsorption despite the expected electrostatic repulsion between the protein and the coating surface.

Much better glycoforms resolution was obtained with a PB-PVS (10%, *m/v*, and 5%, *v/v*, respectively) coating and 20 mM ammonium acetate at pH 8.5, as presented in **Fig. 2.F**. This coating was quite promising due to satisfactory resolutions (R_s ≥1.4) and acceptable efficiencies (N = 30,000 – 125,000). However, despite a careful optimization of the BGE composition and the coating parameters, a stable coating was eventually not obtained and only two consecutive analyses could be satisfactory performed before its deterioration.

The best compromise in terms of glycoforms resolution (R_s ≥1.0), separation efficiency, protein adsorption, and coating stability (RSDs on migration times = 2.0%, *N* = 8) was obtained with a PB-DS coating (10% each) and a BGE composed of 20 mM ammonium acetate at pH 8.5. The obtained electropherogram is shown in **Fig. 2.G**. Disialo- and trisialo-Tf were not baseline resolved but the additional selectivity expected with the MS detection can help for their discrimination according to their different mass-to-charge ratios (*m/z*). The enhanced stability *versus* PB-PVS coating could be explained by a higher average layer thickness for PB-DS than for PB-PVS coating due to the relatively higher molecular size of DS polymer, as recently studied by atomic force microscopy [39].

PB-DS coating was stable to MeOH, MeCN, and 1 M NaOH. Regeneration between runs was not necessary but the coating could be removed with 0.1 M HCl before being coated again, rendering the procedure well adapted for the screening of CDT. Moreover, the sensitivity was equivalent to the one obtained with the CEofix™ CDT procedure, while shorter migration times were provided.

3.1.2 BGE composition

Numerous volatile BGEs composed of acetic acid or formic acid, with or without the presence of ammonium counterions, and at various pH were investigated. TEA was also evaluated for basic BGEs as it has shown interesting results for anionic compounds separation [40].

Among ammonium acetate and formate basic BGEs between 10 and 50 mM, and pH 7.5 to 9.5; as well as TEA between 10 and 25 mM, and pH 8.5 to 11.7; the best resolution between isoforms with a PB-DS coated capillary was obtained with 20 mM ammonium acetate at pH 8.5 (**Fig. 2.G**). This resolution remains inferior to the one obtained with phosphate or borate buffers, which can efficiently interact with silanols or polymers of the capillary surface, leading to decreased secondary interactions between the protein and the surface. Moreover, phosphate can induce a change in the protein net charge but also in its conformation, which can exhibit positive influence on the separation [16].

3.2 CE-ESI-TOF/MS hyphenation

3.2.1 ESI-TOF/MS conditions

After the evaluation of BGE composition and capillary coating in CE-UV configuration, ESI-TOF/MS parameters were investigated *via* Tf infusion through the capillary, including nebulizing gas flow rate, drying gas flow rate and temperature, and ESI and fragmentor voltages. With the sheath-flow interface, consisting in a triple-tube surrounding the capillary outlet and positioned in an orthogonal configuration towards the MS entrance [24], one significant parameter that can affect the protein ionization efficiency is the composition of the sheath liquid. The latter allows for the electrical contact between CE and MS, and an appropriate flow rate for proper ESI process. The hydro-organic composition of the sheath liquid was carefully investigated with different organic solvents (*i.e.*, MeOH, i-PrOH, and MeCN) at different proportions. Although Tf was migrating under anionic form, a positive ESI ionization was selected due to an increased sensitivity compared to negative ionization mode. Thus, a relatively high concentration of acidic solution was added to the sheath liquid to assist the ionization, and its composition and proportion were optimized. **Fig. 3** presents the different intensities observed for the ion $[M+33H]^{33+}$ of tetrasialo-Tf at *ca.* 2411.8 *m/z*. It has to be noticed that the variation of the sheath liquid composition slightly shifted the relative abundance among the multicharged ions, probably due to the unfolding effect of the different organic solvents [28]. The whole ionization profile was therefore monitored during the tuning. The best intensities were obtained with a hydro-organic mixture composed of 50:50 *i*-PrOH-water (*v/v*), while MeOH and MeCN were discarded due to lower signal intensities (**Fig. 3.A**). Higher content of organic solvents were not found advantageous due to unstable ESI currents; while a low proportion led to a poor ionization because of a high surface tension, and, thus, a low volatility. The addition of 5% of formic acid was required to ensure a substantial ionization of the protein (**Fig. 3.B**). Acetic acid gave lower ionization, and higher noise and instability were observed. Trifluoroacetic acid (TFA) was also investigated but led to strong ion suppression.

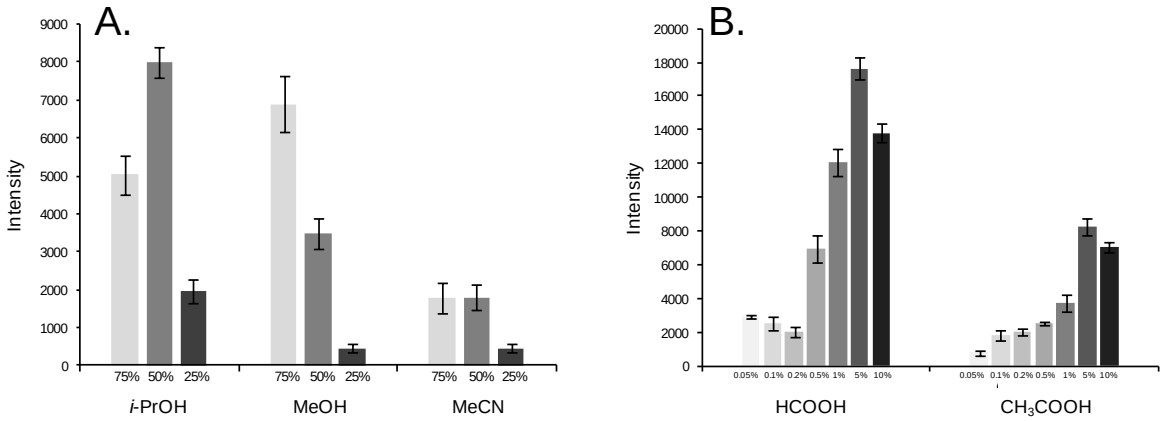

Figure 3. Effect of the sheath liquid composition on the ionization efficiency. The ion $[M+33H]^{33+}$ of tetrasialo-Tf at ca. 2411.8 *m/z* is monitored. A. Proportion of the organic solvent in the sheath liquid, B. Proportion of volatile acids, *i.e.*, formic and acetic acid, in the *i*-ProH-water solution. Error bars express ± 2SD.

With these conditions, a MS profile over the range 2000-3000 *m/z* was observed during Tf infusion, as presented in **Fig. 4.A**. The major and more abundant tetrasialo-Tf was detected with a relatively high charge number ($27 \leq z \leq 37$). Disialo-Tf was also detected with rather low intensities, while other glycoforms were poorly or not detected. The lack of sensitivity was confirmed with deconvoluted mass spectra (**Fig. 4.B**) where tetrasialo-Tf was observed at 79,554 Da (theoretical mass of 79,573 Da), as well as disialo-Tf at 77,214 Da (theoretical mass of 77,365 Da) with a much lower intensity. It is worth mentioning that no standard of each glycoform was available to confirm the TOF/MS detection and glycoform identification with absolute certainty.

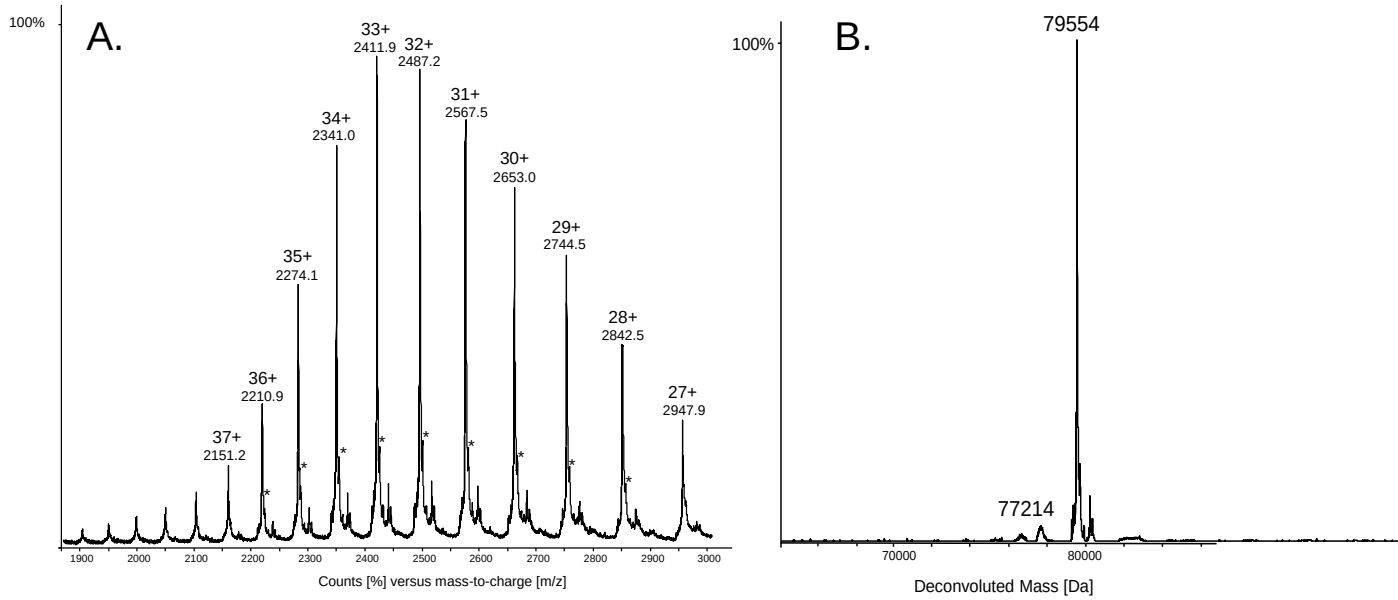

Figure 4. Results obtained with ESI-TOF/MS for Tf infusion. A. MS profile observed for tetrasialo-Tf ($27 \leq z \leq 37$); stars: MS profile of disialo-Tf. B. Deconvoluted mass spectrum. See Section 2.4.2 for experimental conditions**.**

3.2.2 CE-ESI-TOF/MS analysis

The whole methodology was eventually transferred to CE-ESI-TOF/MS on a long capillary with on-line UV detection. With a conventional HD injection of 0.2% of the capillary length (4 nL injected), no peak was observed nor obtained after the algorithmic protein deconvolution, clearly demonstrating a insufficient sensitivity explained by a poor ionization, as illustrated in **Fig. 5.A**. The loading quantity was increased up to 2.7% of the capillary length (68 nL injected) which allowed for the detection of tetrasialo-Tf (**Fig. 5.B.**). Unfortunately, the gain in sensitivity was not sufficient to detect the less abundant and clinically relevant glycoforms. Even one of the most recent hybrid mass analyzer based on triple quadrupole and TOF technologies, providing enhanced performance, led to the same results in terms of sensitivity.

The other issue encountered upon increasing the loading quantity was a loss of glycoforms resolution, confirmed by the online UV detection. This might be due to the loaded quantity during injection and suction effect caused by the nebulizing gas [24]. For the latter, a negative pressure (50 mbar) at the CE inlet during the run did not have any positive impact on the separation efficiency, probably because of the hydrodynamic profile induced. In this context, the use of the sheathless interface could be considered to avoid the sensitivity and resolution issues experienced with the

sheath-flow interface due to the absence of make-up liquid [24]. This prototype proposed by Moini *et al.* [41] with an etched 30-µm i.d. separation capillary already gave interesting results for intact protein analysis [42,43] and should be considered for further experiments.

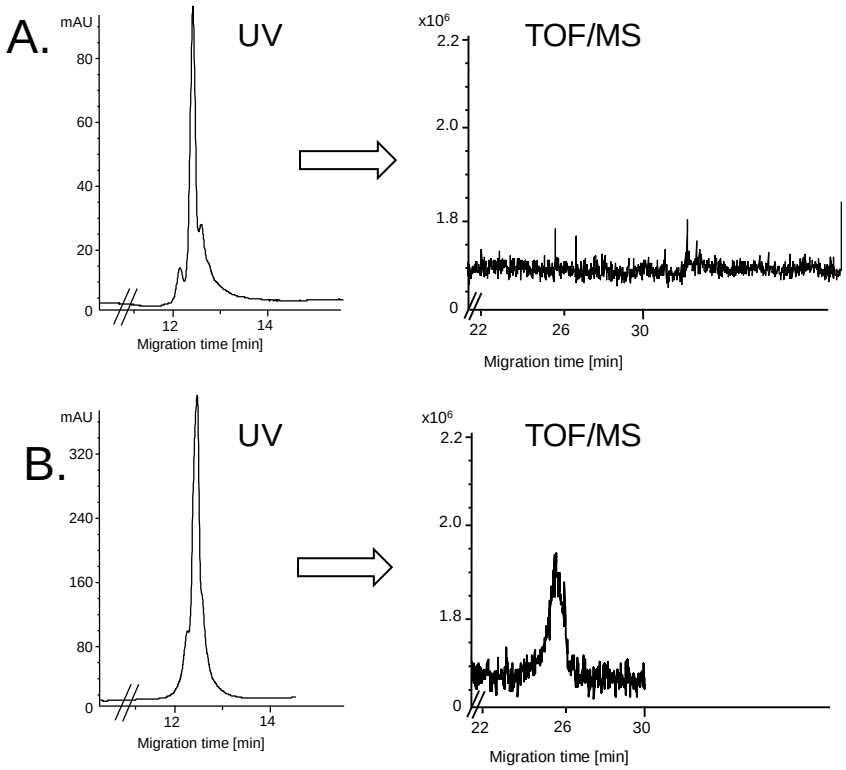

Figure 5. Results obtained with CE-UV-ESI-TOF/MS configuration for different Tf injections. A. Conventional HD injection of 0.2% of the capillary length, B. HD injection of 2.7% of the capillary length. See Section 2.4.2 for experimental conditions.

4 Concluding remarks

CE-UV has been widely used over the last years for the determination of CDT in case of chronic alcohol use evaluation with a commercially available procedure. However, this method presents some limitations in terms of analytical selectivity and sensitivity which could be overcome with MS detection. The method is not MS-compatible due to the presence of a non-volatile BGE and dynamic capillary coating. This study consisted in developing new separation conditions (*i.e.*, BGE composition and coating procedure) to evaluate the Tf glycoforms' separation by CE-ESI-TOF/MS.

Numerous coating compositions and procedures were investigated. The adsorption of heavy proteins to the capillary wall being difficult to predict, very different coatings were tested, *i.e.*, cationic, neutral, and anionic coatings. BGE composition was also optimized for each coating, covering a broad range of pH values. An anionic PB-DS coating (10% each) combined with a BGE composed of 20 mM ammonium acetate at pH 8.5 provided the best results in terms of protein adsorption prevention, glycoforms resolution, efficiency, changes in baseline, migration times' repeatability, and coating stability.

ESI-MS parameters were investigated to enable the ionization and detection of Tf. Tetrasialo-Tf, the more abundant and major isoform, was detected over the range 2000-3000 m/z *via* infusion with a

sheath liquid composed of 50:50 *i*-PrOH-water (*v/v*) with 5% formic acid. Less abundant glycoforms were not detected with these conditions due to poor ionization efficiency.

CE-ESI-TOF/MS experiments brought out the challenges faced for intact protein analysis with a consequent loss of glycoforms resolution inherent to the configuration, as well as poor Tf ionization efficiency. Despite the wide and successful use of the sheath liquid interface for the analysis of low molecular weight compounds, peptides, or light proteins (≤ ~30 kDa) with few post-translational modifications (PTMs), careful geometry improvements should be envisaged, especially in case of rather heavy proteins or even monoclonal antibodies which have intrinsic poor ionization capacity.

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6 Supplementary data

Table 1. Coating procedures. Unless otherwise stated, coating procedures were directly performed within the CE system and were carried out on 64.5-cm long capillaries of 50 µm i.d. The concentration of the polymers, all in aqueous solutions, is expressed in *m/v*, except for PVS which is expressed in *v/v*.

	Cationic coatings		Neutral coating	Anionic coatings				
	PEI	PB-DS-PB	UltraTrol™ LN	CEofix™ CDT kit	CEofix™ MS kit	PB-PVS	PB-DS	PB-DS for CE-MS analysis[1]
Composition	10%	PB: 5% DS: 10%	As provided	As provided	As provided	PB: 10% PVS: 5%	PB: 10% DS: 10%	PB: 10% DS: 10%
Procedure (P = 2 bar)	1M NaOH, 30 min H_2O, 15 min PEI 10%, 10 min Wait, 60 min H_2O, 15 min BGE, 10 min.	1M NaOH, 10 min H_2O, 3 min PB 5%, 10 min Wait, 10 min H_2O, 3 min DS 10%, 10 min Wait, 10 min H_2O, 3 min PB 5%, 10 min BGE, 10 min.	1M NaOH, 10 min H_2O, 10 min UltraTrol™ LN, 20 min BGE, 20 min.	„Conditionner“, 5 min „Rinse“, 5 min „Initiator“, 0.5 min „Buffer“, 5 min	1M NaOH, 5 min H_2O, 5 min Initiator, 0.3 min Accelerator, 0.3 min BGE, 10 min.	1M NaOH, 5 min H_2O, 3.5 min PB 10%, 5 min H_2O, 3.5 min PVS 10%, 5 min H_2O, 3.5 min BGE, 10 min.	1M NaOH, 5 min H_2O, 3.5 min PB 10%, 10 min H_2O, 3.5 min DS 10%, 10 min H_2O, 3.5 min BGE, 10 min.	1M NaOH, 10 min H_2O, 7min PB 10%, 20 min H_2O, 7 min DS 10%, 20 min H_2O, 7 min BGE, 20 min.
Pre-cond. (P = 2 bar)	BGE, 3 min	BGE, 3 min	BGE, 3 min	“Conditionner”, 1 min “Rinse”, 1 min “Buffer”, 3 min	BGE, 3 min	BGE, 3 min	BGE, 3 min	BGE, 6 min
Coating regeneration (P = 2 bar)	-	-	UltraTrol™ LN, 5 min	-	Accelerator, 0.3 min	-	-	-
Storage	Capillary filled with BGE, with tips immersed in BGE-filled vials	Capillary filled with BGE, with tips immersed in BGE-filled vials	Capillary filled with BGE, with tips immersed in BGE-filled vials	Capillary filled with BGE, with tips immersed in BGE-filled vials	Remove the coating with 1M NaOH, 5 min, then H_2O, 5 min Air, 5 min.	Capillary filled with BGE, with tips immersed in BGE-filled vials	Capillary filled with BGE, with tips immersed in BGE-filled vials	Capillary filled with BGE, with tips immersed in BGE-filled vials

[1]: coating procedure for a 129-cm long capillary of 50 µm i.d. Before the coating procedure, capillary outlet was removed from the ESI source

Table 2. Investigated BGEs

BGE	Concentration	pH
Acetic acid	0.5 M	2.6
Acetic acid	1 M	2.4
Acetic acid	2 M	2.2
Formic acid	0.5 M	2.0
Formic acid	1 M	1.8
Ammonium acetate	10 mM (ammonium acetate salt)	7.5; 8.0; 8.5; 9.0; 9.5 (adjusted with NH_4OH)
Ammonium acetate	20 mM (ammonium acetate salt)	7.5; 8.0; 8.5; 9.0; 9.5 (adjusted with NH_4OH)
Ammonium acetate	50 mM (ammonium acetate salt)	7.5; 8.0; 8.5; 9.0; 9.5 (adjusted with NH_4OH)
Ammonium formate	10 mM (ammonium formate salt)	7.5; 8.0; 8.5; 9.0; 9.5 (adjusted with NH_4OH)
Ammonium formate	20 mM (ammonium formate salt)	7.5; 8.0; 8.5; 9.0; 9.5 (adjusted with NH_4OH)
Ammonium formate	50 mM (ammonium formate salt)	7.5; 8.0; 8.5; 9.0; 9.5 (adjusted with NH_4OH)
TEA	10 mM	11.5
TEA	25 mM	11.7
TEA	25 mM	8.5 (adjusted with acetic acid)

Chapter V.

Chapter V. Conclusions and perspectives

The present thesis aimed at presenting some methodological improvements in CE-MS to enhance the overall sensitivity, allowing for its use in clinical and forensic toxicology. Although showing a history of more than 20 years, CE-MS is poorly accepted as a competitive analytical tool and suffers from a lack of interest of the toxicologists' community. Indeed, CE-MS is considered complex to handle and not enough sensitive compared to GC-MS or LC-MS for the analytical questions encountered in toxicology. Furthermore, CE-MS is not recognized as a high-throughput or high-robust technique, both items being substantial in daily clinical or forensic use. These statements mostly rely on a lack of knowledge of the basic principle and practical considerations of the technique. When correctly handled and if the entire capacities of the coupling are invested, CE-MS can be completely integrated in the daily used analytical equipment in clinical and forensic laboratories, as shown in the present manuscript.

The years 2000 saw an expanded consciousness in the development of green chemistry techniques and methodologies to protect both health and environment. From this sustainable concern emerged numerous attempts in decreasing the solvent quantities or replacing them by less-toxic alternatives. In this context, the combination of miniaturized sample pre-treatment techniques is considered very attractive. Using microextraction techniques combined to CE analysis provides a low consumption of organic solvents. Not only is this combination considered green, but it also shows promising performance in terms of sensitivity enhancement. Preconcentration factors higher than 100 were obtained with the dispersive liquid-liquid microextraction developed in this work, and the procedure combined with CE-MS analysis led to limits of detection lower than the ng/mL range for a large set of drugs of abuse in urine samples. Until now, numerous applications of miniaturized sample preparation in combination with CE-UV analysis have been proposed, but very few studies underscored the interest of coupling microextractions with CE-MS. The future will likely see more applications, mainly *via* on-line or in-line configurations, the latter allowing for a complete automation of the procedure, and, thus, high-throughput analyses.

Even with these miniaturized approaches, which not only decrease the solvents consumption but also the extraction time, the easiest sample pre-treatment is frequently preferred due to the time limitations (*e.g.*, in clinical departments) or the need for generic methods. Multi-target or general unknown screening procedures aim at detecting the highest number of compounds which show relatively large differences in their physico-chemical properties. In this context, a simple dilution of the sample is often preferred prior to the injection. In CE-MS, diluting the sample prior to the injection without further ado is not recommended due to the already quite low injected volumes. Nevertheless, based on its separation principle, CE presents the advantage of possible on-line preconcentration to strongly enhance the loading quantity. This approach was selected in this work for the development of a multi-target screening assay by CE-ESI-TOF/MS followed by confirmation/quantitation with CE-MS/MS. Both methods involved a simple urine acidification prior to the injection with a pH-mediated stacking procedure. In order to be fully competitive in toxicological fields, the methods were developed by considering each part of the analytical process to provide a highly sensitive, high-throughput, and high-resolution screening and a sensitive, selective, and accurate quantitation. The complete procedure was proposed as a two-step workflow

but each method may be complementary to immunoassays, GC-MS, or LC-MS, providing an interesting and legally required orthogonal selectivity for confirmation purposes.

The field of toxicology most frequently involves the determination of low-molecular weight compounds such as xenobiotics and their metabolites. Nevertheless, oligopeptide and protein determination is also of utmost interest in clinical fields, including the diagnosis opportunities offered by the detection of biomarkers (*e.g.*, troponin I in acute myocardial infarction, fibulin 3 peptides in osteoarthritis, amyloid β peptides in Alzheimer's disease, *etc.*); or for forensic purposes in case of doping control (*e.g.,* EPO, hemoglobin-oxygen based carriers, selective androgen receptor modulators, *etc.*) and in the context of chronic alcohol abuse diagnosis and monitoring, as illustrated in this work. Due to its separation principle, CE-MS is particularly well suited for the analysis of intact proteins, providing a very high theoretical plates number. Nevertheless, if the analytical challenge is complex (*e.g.*, large proteins, numerous isoforms, or complex biological matrix), multiple issues can be encountered, including those observed and described in this work. The development of a CE-MS method for the determination of carbohydrate-deficient transferrin highlighted the issues related to protein adsorption, poor ionization, modifications of the glycoforms pattern with method transfer, *etc*. Nevertheless, the same CE-ESI-TOF/MS configuration used in this project had previously shown its applicability to the determination of multiple intact proteins (insulin, human growth hormone, hemoglobin-oxygen based carriers, *etc*.). All projects converge to the statement that each protein shows a different analytical behavior, requiring an adapted and dedicated method development.

Today, CE-MS is most frequently used in niche applications where neither LC-MS nor GC-MS showed substantial performance, including enantioselective determination or intact protein analysis. Furthermore, due to the speculated complexity of the coupling, other applications remain confined to academic laboratories. This thesis and the related studies are thus expected to give an overview of the extended capacities of CE-MS and its advantages which are undoubtedly useful in addition to the widely used chromatographic techniques in multiple clinical and forensic applications.

A more widespread use of CE-MS may be further encouraged by improving the core of the combination, *i.e.*, the interface. At present, only one interface is commercially available which provides a good robustness but suffers from a lower sensitivity compared to the alternative prototype. From a best-case-scenario viewpoint, the sheathless interface should be rapidly commercialized to widen the choice in interface selection, provided that its robustness is sufficient. Today, selecting the adapted interface relies on "sensitivity *versus* robustness". Choosing the robustness while attempting to enhance the sensitivity, considering the whole analytical process, was the opted strategy in the presented studies. Nevertheless, there is an urgent need in improving both interfaces to avoid complex and time-consuming method developments and lead to the commercialization of *plug-and-play* configurations that may be easily used by analytical expert as well as technicians. The solution presumably relies on the development of low-flow-based interfaces, keeping the advantages of the sheath liquid (stability, ionization tuning, CE-MS decoupling) but at flow rates of hundreds of nL/min. In this context, the implementation of CE-MS in clinical or forensic laboratories will be probably pushed along.

Chapter VI.

Chapter VI. Appendices

Appendix I

Sample preparation of urine samples prior to CE-MS in toxicological analysis

M. Rovini, J. Schappler, I. Kohler, M. Anzini, J.L. Veuthey, S. Rudaz, J Chem Chem Eng 5 (2011) 583

Appendix II

Microextraction liquide-liquide dispersive combinée à l'électrophorèse capillaire et la spectrométrie de masse pour l'analyse de l'urine

I. Kohler, J. Schappler, T. Sierro, S. Rudaz, CJ'MAG 4 (2012) 3

Appendix III

Single-run separation of closely related cationic and anionic compounds by CE-ESI-MS: application to the simultaneous analysis of melamine and its analogs in milk

I. Kohler, E. Cognard, I. Marchi, D. Ortelli, P. Edder, J.L. Veuthey, S. Rudaz, J. Schappler, Chimia 65 (2011) 389

Appendix IV

Compatibility of Agilent Jet Stream thermal gradient focusing technology with CE/MS

I. Kohler, J. Schappler, S. Rudaz, H.P. Zimmermann, C. Wenz, Agilent Technologies Technical Note, publication number 5990-9716EN (2012)

Appendix V

Highly sensitive CE-ESI-MS/MS for accurate quantitation of drugs of abuse in bioanalysis using the Agilent 6490 Triple Quadrupole LC/MS System

I. Kohler, J. Schappler, S. Rudaz, M. Greiner, Agilent Technologies Application Note, publication number 5991-2395EN (2013)

Appendix I.

Sample preparation of urine samples prior to CE-MS in toxicological analysis

M. Rovini, J. Schappler, I. Kohler, M. Anzini, J.L. Veuthey, S. Rudaz, J Chem Chem Eng 5 (2011) 583

J. Chem. Chem. Eng. 5 (2011) 583-594

Sample Preparation of Urine Samples Prior to CE-MS in Toxicological Analysis

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Abstract: Capillary electrophoresis (CE) coupled with mass spectrometry (MS) with a sheath liquid interface is nowadays recognized as a powerful separation technique for drugs and metabolites analysis in human urine and can be applied in numerous fields such as clinical toxicology, drug substitution monitoring, forensic sciences and antidoping. With an acidic background electrolyte containing 15 mM ammonium formate at pH 2.5 and a sheath liquid consisting in a mixture of isopropanol/water (50:50, v/v) with 0.5% formic acid, CE-ESI-MS in positive mode demonstrated excellent performance for simultaneous analysis of basic drugs of abuse and metabolites in urine (e.g. cocaine, amphetamine, morphine and phase II metabolites). To achieve the desired level of sensitivity, two injection modes and three sample pre-treatments were evaluated. The detection of basic drugs and phase II metabolites in diluted urine was achieved at concentrations above 1 µg/mL. In order to enhance sensitivity, a sample preparation was required. A liquid-liquid extraction (LLE) was compared with solid-phase extraction. LLE was performed at alkaline pH and samples were electrokinetically injected. A chemometric approach (Doehlert design) was carried out in order to determine optimized injection parameters. Limits of detection (LOD) down to 10 ng/mL were reached with field-amplified sample injection but phase II metabolites were not extracted. Therefore, instead of LLE a SPE was performed on C18 sorbent, and elution fraction after washing step containing phase II metabolites was loaded on mixed-mode anion exchanger cartridges. After electrokinetic injection, this two-step SPE allowed LOD ca. 10 ng/mL for drugs and phase II metabolites.

Key words: Capillary electrophoresis, mass spectrometry, sample preparation, urine, toxicology.

Abbreviations List

6-MAM	6-monoacetylmorphine
ACN	Acetonitrile
BE	Benzoylecgonine
BGE	Background electrolyte
C6G	Codeine-6-glucuronide
CE	Capillary electrophoresis
DET	Diethyltryptamine
EG	Ecgonine
EK	Electrokinetic injection
EME	Ecgoninemethylester
ESI	Electrospray ionization
FASI	Field-amplified sample injection
FS	Fused silica
HD	Hydrodynamic injection
i-prOH	Isopropanol
LC	Liquid chromatography
LLE	Liquid-liquid extraction
LOD	Limit of detection
M3G	Morphine-3-glucuronide
MA	Metamphetamine
MDA	3,4-methylenedioxyamphetamine
MDEA	3,4-methylenedioxyethylamphetamine
MDMA	4-methylenedioxymetamphetamine
MeOH	Methanol
MS	Mass spectrometry
SPE	Solid-phase extraction

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1. Introduction

Monitoring of drugs of abuse and illicit substances is a timely topic in human toxicology, supervision of drug

substitution, forensic sciences, workplace drug testing and antidoping. In this context, urine is particularly well adapted to determine those drugs since it is easily available in large quantities and non-invasive to collect [1-3]. Furthermore, urine allows a relatively large detection window for xenobiotics and/or metabolites. To analyze those drugs, numerous immunoassays and chromatographic methods have been developed. Immunological techniques are attractive because of their ease of performance, speed of analysis and sensitivity, they are thus often employed for screening purposes [4-7]. Chromatographic methods coupled with various detectors are typically used for confirmatory testing. For drug substances, liquid chromatography (LC) is considered as the method of choice since satisfactory results are obtained without a pre-derivatization step unlike gas chromatography. Therefore, it has been demonstrated that liquid chromatography coupled to mass spectrometry (MS) operating in the single or tandem mode (LC-MS or LC-MS/MS) is particularly well adapted for the analysis of drugs in toxicological and forensic fields, MS detection generally providing information on substance identity, with lower detection limits and less interferences, compared to immunoassays [8-13].

During the past decade, electrophoretic separations in fused-silica (FS) capillaries were also found suitable for drug monitoring in body fluids [14-16]. A comprehensive concept for toxicological drug screening and confirmation by capillary electrophoresis (CE) has been developed and successfully applied to the monitoring of drugs of abuse in urine samples [17, 18]. In analogy to GC-MS and LC-MS, hyphenation of CE with MS (CE-MS) is an attractive approach to gather structural information of compounds [19, 20]. CE-MS was reported for the determination in urine of haloperidol [21], non-steroidal anti-inflammatory drugs and some of their metabolites [22], paracetamol and metabolites [23,24], non-opioid analgesics and metabolites [25], and mainly geared towards the elucidation and confirmation of drug metabolism.

In this work, a generic acidic aqueous background electrolyte (BGE) containing 15 mM ammonium formate at pH 2.5 was used for CE-MS with electrospray ionization (ESI) in the positive mode and demonstrated excellent performance for the simultaneous analysis of a large number of basic drugs of abuse in spiked urine, including cocaine and metabolites, amphetamine and derivatives, morphine and analogs, as well as other compounds of pharmaceutical interest. Furthermore, the possibility to simultaneously detect very polar analytes such as phase II metabolites (e.g. glucuronides) was investigated with model compounds. Three sample preparations, namely urine dilution, liquid-liquid extraction (LLE) in alkaline conditions with back extraction and solid-phase extraction (SPE) on different sorbents were tested and compared in terms of selectivity, sensitivity and time delivery constraints.

2. Material and Methods

2.1 Chemicals

Dextromethorphan, ephedrine, norephedrine and imipramine were purchased from Sigma (St. Louis, MO, USA). Papaverine, pethidine, methadone, morphine, codeine, amphetamine (A), noscapine and ethylmorphine were provided by Siegfried (Zofingen, Switzerland). Fentanyl was obtained from Sintetica (Mendrisio, Switzerland). Methamphetamine (MA), 3,4-methylenedioxyamphetamine (MDA), 4-methylenedioxymethamphetamine (MDMA, Ecstasy), 3,4-methylenedioxyethylamphetamine (MDEA), 6-monoacetylmorphine (6-MAM), ecgonine (EG), ecgoninemethylester (EME), benzoylecgonine (BE), cocaine and norcocaine were purchased from Lipomed (Arlesheim, Switzerland). Morphine-3-glucuronide (M3G) and codeine-6-glucuronide (C6G) were obtained from Lipomed (Cambridge, MA, USA). 6-acetylcodeine, pseudomorphine, thebaine, N, N-diethyltryptamine (DET), and mescaline were a gift from the University Center of Legal Medicine (CURML, Lausanne-Geneva,

Switzerland). Ammonium formate, formic acid, ethyl acetate, methanol (MeOH), isopropanol (*i*-prOH), chlorobutane and chloroform were of analytical reagent grade from Fluka. Ultrapure water was supplied by a Milli-Q gradient A10 purification unit from Millipore (Bedford, MA, USA).

2.2 Background Electrolyte (BGE) and Standard Solutions

The BGE consisted of a 15 mm (ionic strength) ammonium formate buffer set at pH 2.5. Stock standard solutions of analytes were prepared by dissolving each compound in MeOH to obtain a concentration of 1 mg/mL and stored at 4 °C until use. Standard solutions at desired concentrations were prepared daily by diluting stock solutions in water.

2.3 Sample Preparation Procedures

Urine pre-treatments included: (1) dilution; (2) liquid-liquid extraction (LLE); and (3) solid-phase extraction (SPE).

(1) Dilution was achieved by mixing urine with an equal volume of BGE (1:1, v/v);

(2) A double LLE was performed. 200 µL of a 50 mM borate buffer at pH 9 was added to 200 µL of spiked urine. 400 µL of ethyl acetate was added to this solution. After vortex-mixing for about 60 s, 200 µL of the organic phase was transferred into a vial containing 100 µL of 0.001 N HCl at pH 3. After shaking for about 60 s, the aqueous phase was transferred into a vial and diluted with MeOH (1:10, v/v);

(3) SPE was performed using disposable, hydrophilic-lipophilic balance copolymer cartridges (OASIS® HLB, Waters) with a Vac-Elut setup (Varian, Palo Alto, Ca, USA). Briefly, the cartridges were conditioned with 2 mL of MeOH and 2 mL of a 50 mM borate buffer at pH 9 using vacuum aspiration. 1 mL of urine was added to 1 mL of a 50 mM borate buffer at pH 9. This solution was loaded onto a cartridge. Washing steps were sequentially performed with 1 mL of water and 500 µL of MeOH-formic acid (98:2, v/v) applying vacuum aspiration.

To extract M3G and C6G, a double SPE was achieved firstly with C18 cartridges (Varian) and secondly with mixed-mode anion exchanger OASIS® MAX cartridges (Waters, Milford, USA). Briefly, C18 cartridges were conditioned with 2 mL of MeOH and 2 mL of a 50 mM borate buffer at pH 9 using vacuum aspiration without drying the sorbent bed. 1 mL of urine was added to 1 mL of borate buffer (pH 9; 50 mM). This solution was loaded slowly and the cartridge was then washed with 3 mL of H_2O-MeOH (90:10, v/v) applying vacuum aspiration. The eluate containing M3G and C6G was collected in a glass tube and added to 3 mL of a 50 mM borate buffer at pH 9. This solution was loaded onto an OASIS® MAX cartridge and slowly drawn through. This cartridge was washed with 1 mL of water applying vacuum aspiration. Finally, both C18 and OASIS® MAX cartridges were eluted with 500 µL of MeOH-formic acid (98:2, v/v) applying vacuum aspiration.

2.4 CE-ESI-MS Instrumentation

CE experiments were performed with a HP ^{3D}CE system (Agilent, Waldbronn, Germany) equipped with an on-capillary diode array detector, an autosampler, and a power supply able to deliver up to 30 kV, and hyphenated with a 1100 MSD single quadrupole (Agilent, Palo Alto, CA, USA). Separation was performed in an uncoated FS capillary (BGB Analytik AG, Böckten, Switzerland) with a total length of 64.5 cm and an internal diameter of 50 µm. Hydrodynamic (HD) injection was performed by applying pressure at the inlet (50 mbar) for 10 s (1.1% of capillary length) while electrokinetic (EK) injection was performed by applying voltage at the inlet (15 kV) for 50 s. Experiments were carried out in positive polarity mode (anode at the inlet and cathode at the outlet). A constant voltage of 30 kV was applied during the analysis with an initial ramping of 1000 V/s (30 s). Before its first use, the capillary was sequentially rinsed with MeOH, NaOH 0.1 N, water, and BGE at 1 bar for 5 min each. The capillary was daily conditioned with MeOH and

water at 2 bar for 2 min each. Prior to each sample injection, the capillary was rinsed at 2 bar for 1 min with fresh BGE. When not in use, the capillary was rinsed with water and then dry-stored. Since the electrophoretic process alters the running buffer pH by electrolysis and subsequently changes migration times, separation buffer was refreshed every four runs.

In order to hyphenate the CE instrument with the mass spectrometer, a CE-ESI-MS adapter kit sheath liquid interface from Agilent was used. The sheath liquid was an *i*-prOH-water mixture (50:50, v/v) containing formic acid (0.5%, v/v) and delivered by a MilliGAT model M6 micropump (Vici AG, Switzerland) at 3 µL/min. ESI capillary and fragmentor voltages were set at +4500 V and 70 V, respectively. Nebulizing pressure and drying gas flow rate were respectively set at 4 psi and 4 L/min. Drying gas temperature was set at 250 °C. These values were selected according to Geiser et al. [26]. MS detection was carried out in SIM mode for the positive molecular ion $[M+H]^+$ for each compound.

2.5 Softwares

BGE solutions were prepared with the help of PHoEBuS software (version 1.3, Analis, Namur, Belgium). CE ChemStation (version A.02.10, Agilent, Waldbronn, Germany) was used for instrument control, data acquisition and data handling. Experimental design as well as responses surface drawing was achieved with NEMROD (LPRAI, Marseille, France).

3. Results and Discussion

Thirty compounds were chosen to include representative of basic compounds found in toxicology, as well as two important phase II metabolites (*i.e.*, glucuronides). Analytes were sort out in five distinct classes according to their physico-chemical and pharmacological properties (Table 1).

Groups were divided between cocaine and metabolites, amphetamine and derivatives, morphine and analogs, other pharmaceutical compounds, and phase II metabolites. The latter can be found at relatively high concentration in urine and can be quite difficult to evidence when reversed phase liquid chromatography is employed, according to their relatively high polarity. Therefore, to simultaneously analyze these drugs and metabolites in urine by CE-ESI-MS, several sample preparations were evaluated and discussed regarding the sensitivity level achieved, as well as the time delivery constraints.

3.1 Dilute and Shoot

An easy and fast way to analyze urine samples consists of directly injecting diluted urine aliquots into CE capillary. This approach, limited when UV detection is used due to the lack of selectivity and sensitivity, could be envisaged whith MS detection, taken into account the additional selectivity and sensitivity afforded by this detector. Preliminary experiments performed with spiked urines and ESI-MS detection showed that urines had to be diluted with BGE (1:1, *v/v*) prior to injection to provide narrow peaks. CE-ESI-MS of two-fold diluted urine containing drugs at 5 µg/mL each was performed. All compounds, except BE, were detected as $[M+H]^+$ species (Fig. 1).

The limits of detection (LOD), estimated with a signal-to-noise ratio of 3, were found around 1 µg/mL in urine, considering a classical hydrodynamic (HD) injection (50 mbar for 10 s, equivalent to 1.1 % of capillary length). This injection mode was used because of its good reliability and simplicity and the sample quantity loaded into the capillary remained nearly independent on the sample matrix. Indeed, final samples could present conductivity and ionic strength differences due to the important matrix variability when dealing with urine samples.

The behavior of the selected glucuronides was independently investigated according to their particular physico-chemical properties, since M3G and C6G, unlike free compounds, are highly hydrophilic. Both

Table 1 Drugs of abuse and their physico-chemical properties.

Compounds	MM	pK_a (a/b)*	logD (pH9)*
Cocaine and metabolites			
Ecgonine (EG)	185.2	3.60[a] / 11.43[b]	-3.10
Ecgoninemethylester (EME)	199.3	9.57[b]	-0.90
Benzoylecgonine (BE)	289.3	3.35[a] / 10.82[b]	0.21
Norcocaïne	289.3	9.02[b]	2.47
Cocaine	303.4	8.97[b]	2.79
Amphetamine and derivatives			
Amphetamine (A)	135.2	9.94[b]	0.82
Methamphetamine (MA)	149.2	10.38[b]	0.55
Norephedrine	151.2	8.47[b]	0.68
Ephedrine	165.2	9.38[b]	0.52
3,4-mehylenedioxyamphetamine (MDA)	179.2	9.94[b]	0.68
3,4-methylenedioxymethamphetamine (MDMA, Ecstasy)	193.2	10.32[b]	0.47
3,4-methylenedioxyethylamphetamine (MDEA, Eve)	207.3	10.34[b]	0.99
Mescaline	211.3	9.56[b]	0.23
Morphine and analogs			
Dextromethorphan	271.4	9.13[b]	3.74
Morphine	285.3	9.50[a] / 8.26[b]	0.23
Codeine	299.4	8.25[b]	1.19
Thebaine	311.4	7.69[b]	2.49
Ethylmorphine	313.4	8.25[b]	1.66
6-monoacetylmorphine (6-MAM)	327.4	9.46[a] / 7.96[b]	1.13
6-acetylcodeine	341.4	8.03 [b]	2.05
Pseudomorphine	568.7	8.51[a] / 6.94 [b]	-1.23
Other substances			
N,N-diethyltryptamine (DET)	216.3	10.54[b]	1.58
Pethidine	247.3	8.58[b]	2.21
Imipramine	280.4	9.49[b]	4.19
Methadone	309.5	9.05[b]	3.87
Fentanyl	336.5	9.06[b]	3.55
Papaverine	339.4	6.32[b]	3.74
Noscapine	413.4	6.32[b]	2.82
Phase II metabolites			
Morphine-3-glucuronide (M3G)	461.5	2.78[a] / 8.10[b]	-5.25
Codeine-6-glucuronide (C6G)	475.5	2.79[a] / 8.10[b]	-4.12

* pK_a and $logD^{pH9}$ values calculated using Advanced Chemistry Development software version 8.14 for Solaris (ACD/Labs, Toronto, Canada). [(a)]: acidic pK_a / [(b)]: basic pK_a.

possess a negative logP (-0.8 and -1.6, respectively), an acidic pK_a (2.8) and a basic pK_a (8.1) [27]. Hence, separation with an acidic BGE could be achieved, because at pH 2.5, M3G and C6G were globally positively-charged. The "dilute and shoot" methodology was applied to urine samples containing morphine, codeine, and their respective phase II glucuronated metabolites as presented in Fig. 2. LOD were around 5 µg/mL for glucuronides in urine.

Therefore, the "dilute and shoot" approach combined with conventional HD injection is convenient to rapidly analyze basic drugs and metabolites at ppm level in urine samples. In order to enhance sensitivity, the biological material has to be selectively prepared and compounds extracted from urine. For this purpose, LLE and SPE procedures were investigated.

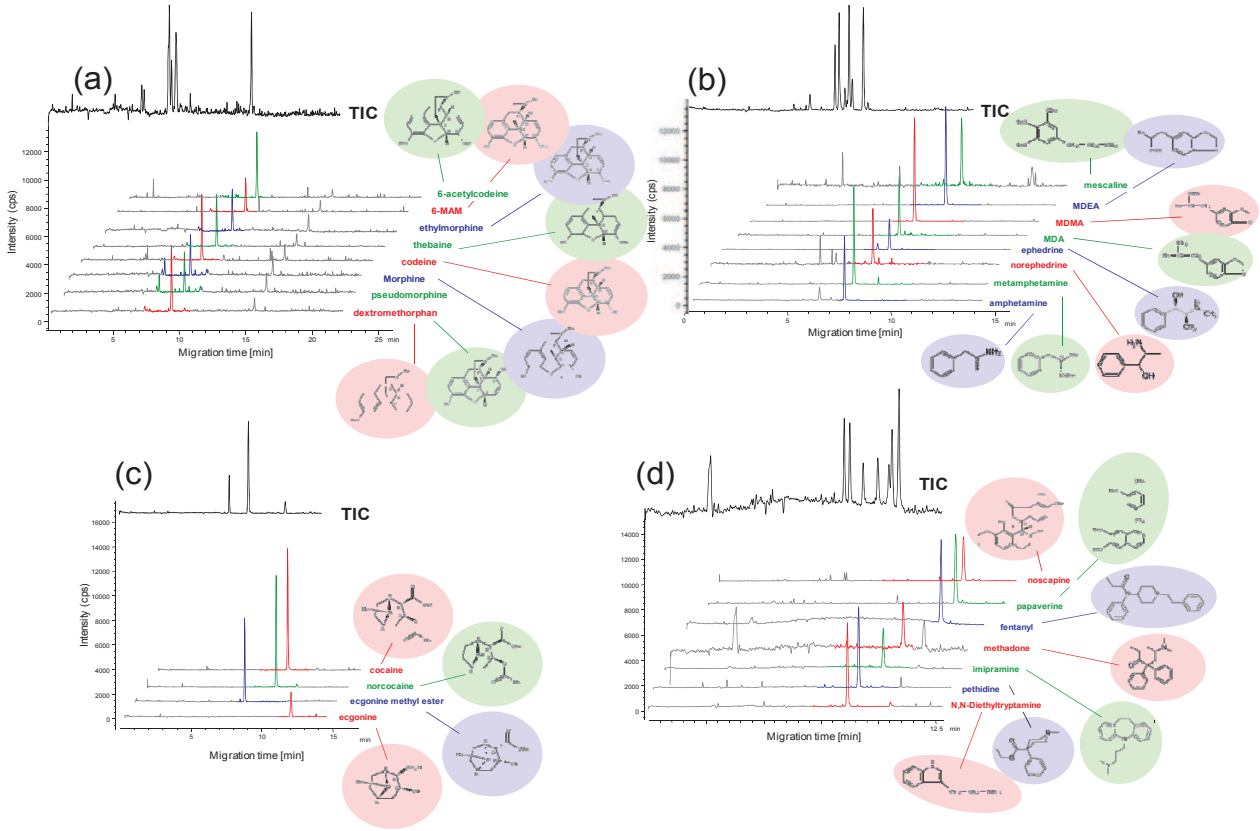

Fig. 1 CE-ESI-MS electropherograms obtained after two-fold dilution of fortified urine containing (a) Morphine and analogs (1 µg/mL each); (b) Amphetamine and derivatives (1 µg/mL each); (c) Cocaine and metabolites (1 µg/mL each); (d) Other drugs (1 µg/mL each). Capillary: FS, 64.5 cm, 50 µm ID; BGE: 15 mM ammonium formate, pH 2.5; Separation conditions: 25 °C, +30 kV (0.5 min ramping); HD injection: 50 mbar for 10 s; sheath liquid: *i*-prOH-water (50:50, *v/v*) + 0.5% formic acid, 3 µL/min; Drying gas: 250 °C, 4 L/min; Nebulizing gas: 4 psi; ESI voltage: +4500 V; Fragmentor voltage: 70 V.

3.2 Liquid-Liquid Extraction with Electrokinetic Injection

Liquid-liquid Extraction (LLE)

According to the physico-chemical properties of tested compounds, LLE at alkaline pH was chosen. In order to obtain maximum recovery and sample purity, as well as to directly inject the extract, a back-extraction in acidic aqueous solution was considered. All steps of the LLE were investigated, including the nature of the organic solvent, the pH of both initial and back-extractions, as well as the solvent used for final reconstitution. Several extraction mixtures were evaluated and ethyl acetate was found appropriate because of the wide polarity range of investigated compounds.

The maximal recovery of the first extraction was obtained at pH 9 because morphine, which possesses an acidic pK_a (9.5) and a basic pK_a (8.3), was approximately neutral (ca. 80%) at this pH value, as desired for a successful quantitative extraction. This pH was also considered as a good compromise for the extraction of 6-MAM (which possesses an acidic pK_a (9.5) and a basic pK_a (8.0)) and pseudomorphine (acidic pK_a (8.5) and basic pK_a (6.9)).

The organic phase was transferred into a vial containing an acidic solution at various concentrations. Starting with pure water, various back-extraction solutions were tested with an increase of HCl concentration. The latter was investigated in terms of extraction recovery but also to allow electrokinetic (EK) injection. It is well known that in CE, EK injection allows enhanced sensitivity (up to 1000-fold) compared to conventional HD injection

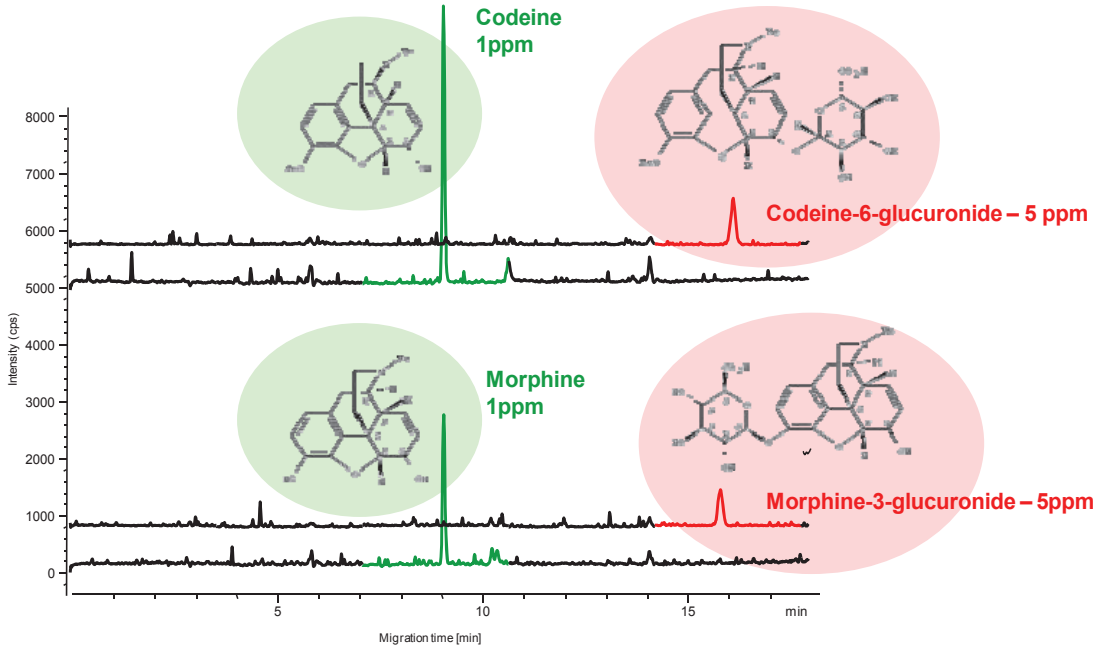

Fig. 2 CE-ESI-MS electropherograms obtained after two-fold dilution of fortified urine containing morphine, codeine (1 µg/mL each) and their respective glucuronides (5 µg/mL each). Experimental conditions as in Fig. 1.

because of achievable on-line sample pre-concentration [28-30]. With a high concentration of HCl (0.1 N, pH 1), the EK injection (15 kV for 50 s) did not give interesting results, because ion concentration was too high and competed with the analyte during the injection. On the other hand, a low concentration of HCl (0.0001 N, pH 4) allowed a maximal pre-concentration, closed to that obtained when analytes are diluted with pure water. However, this acid concentration was eventually not sufficient to quantitatively back-extract analytes from the organic phase. An intermediate concentration of HCl (0.001 N, pH 3) was selected as the best compromise between maximal recovery (>75%) and the possibility to benefit from EK injection.

Because sample dilution in organic solvent (mainly acetonitrile or MeOH) has already demonstrated to produce stacking effect due to the high field strength obtained by the low sample zone conductivity [28, 30, 31], the back-extracted sample was further diluted with MeOH (1:10, v/v). A pre-plug of water (50 mbar for 3 s, equivalent to 0.3% of the capillary length) was also included as described in FASI experiments, which greatly enhanced efficiency and consequently sensitivity [30-32].

Electrokinetic Injection (EK)

Experiments were first achieved with short injection times (5-15 s) at relatively low voltages (2.5-5 kV). Under these conditions, obtained electropherograms were of relatively low quality, with poor peak shapes. Indeed the sample conductivity was variable between samples and afforded differences in voltage drop and analyte quantity loaded during the injection [33]. Because the injection conditions were mainly related to two experimental parameters which are interacting, namely injection time (X1) and voltage (X2), a chemometric approach based on a second degree design was employed. As analytical response, the mean peak area obtained for a representative compound of each analyte group was monitored. Because the initial range could potentially not contain the optimal conditions, a Doehlert design was selected, offering a uniform distribution of points over the whole experimental region, arranged in a rhomboidal figure. Doehlert designs present some interesting features, including the possible extension to any direction of the

experimental domain. New designs may be constructed overlapping with the old design and requiring only three additional experiments.

Initial experiments were achieved with values of 10 to 50 s for injection time and 5 to 15 kV for injection voltage. The prediction quality of the model was checked with statistical tools, including R2 and Q2. The obtained response surface is presented in Fig. 3(a). As expected, the simultaneous increase in injection time and voltage led to a maximal sensitivity. However, because the optimal response was not included in the investigated range, an extension of the experimental domain driven by the initial plan results was achieved to higher values of injection time (80 s) and voltage (20 kV). As presented in Fig. 3(b), an optimal value represented by an important curvature of the response surface was reached with an injection of 15 kV for 50 s.

In these conditions and as presented in Fig. 4, LOD down to 10 ng/mL were reached for each tested analyte. It has to be noted that several assays were conducted for both glucuronides but the developed LLE-EK procedure was not appropriate because phase II metabolites were not extracted considering their high polarity. Therefore, LLE extraction prior to EK injection gave an important level of selectivity and sensitivity. Very low detection levels were reached (10 ng/mL), but the sample preparation remained time consuming and only compounds with a relatively low polarity values could be easily extracted.

3.3 Solid-Phase Extraction (SPE)

Single SPE

Assays with SPE were performed considering different types of sorbents: (1) a pure hydrophobic sorbent (C18); (2) a hydrophilic-lipophilic polymer (OASIS® HLB); and (3) a mixed-mode strong anionic exchanger sorbent (OASIS® MAX). They were selected because of their potentiality to extract both free and conjugated drugs, and were compared in terms of extraction yields and sample quality for MS detection. Each sample was injected in CE-MS using FASI, which involved a pre-plug of water (50 mbar for 3 s, equivalent to 0.3% of the capillary length) and a subsequent sample EK injection (15 kV for 50 s).

With the hydrophilic-lipophilic polymer (OASIS® HLB), the LOD of most compounds was around 50

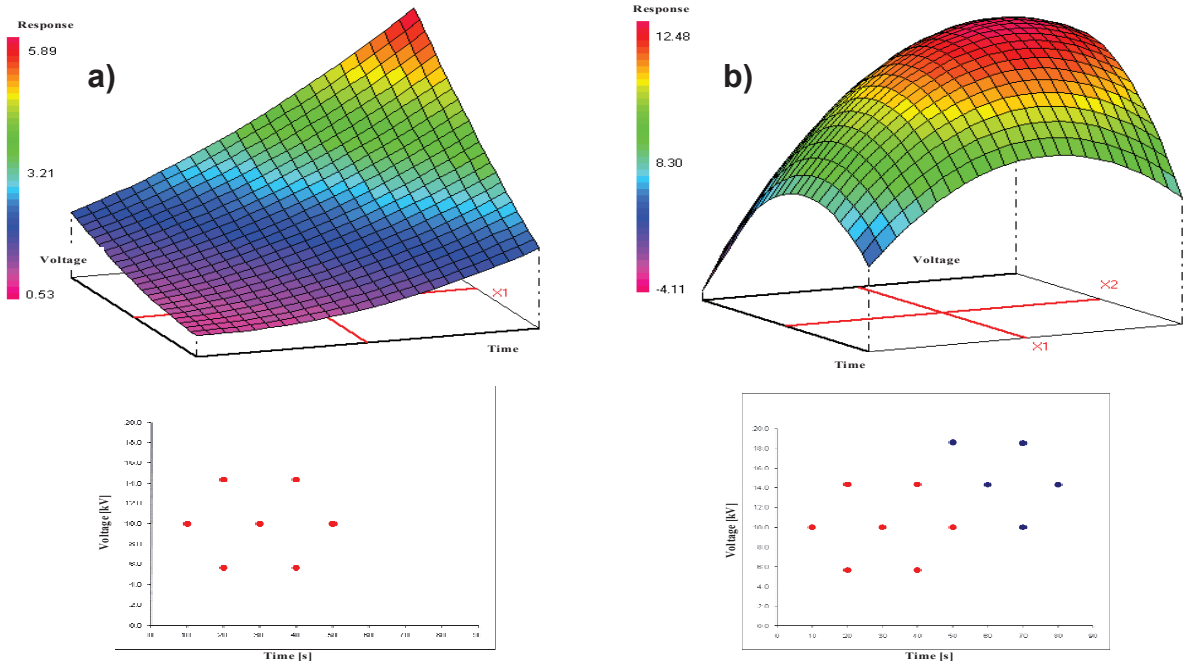

Fig. 3 Response surfaces and experimental domains for optimizing EK injection. (a) step 1 (5-15 kV for 10-50 s) and (b) step 2 (up to 20 kV and 80 s). See text for experimental conditions.

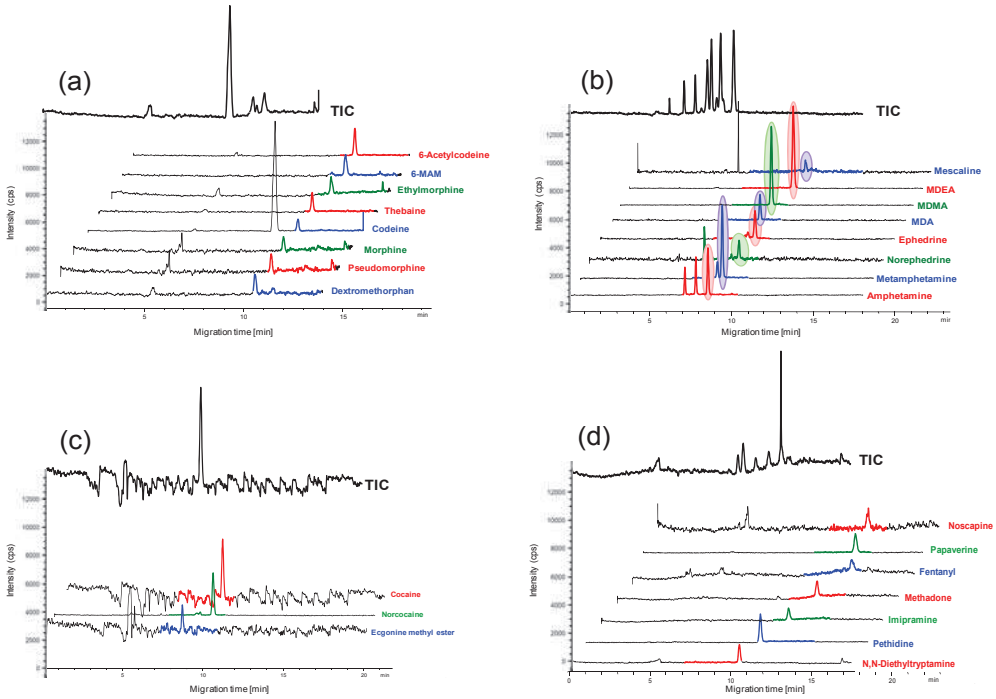

Fig. 4 CE-ESI-MS electropherograms obtained after liquid-liquid double extraction of fortified urine containing (a) Morphine and analogs (10 ng/mL each); (b) Amphetamine and derivatives (10 ng/mL each); (c) Cocaine and metabolites (10 ng/mL each); (d) Other drugs (10 ng/mL each). FASI: 50 mbar for 3 s of water followed by 15 kV for 50 s of sample. Other experimental conditions as in Fig. 1.

ng/mL (Fig. 5), thanks to both SPE and FASI. As expected with this sorbent, conjugated analytes were also retained and finally eluted with 500 µL of acidic MeOH. Concentrations as low as 10 ng/mL in urine were reached with this procedure.

The major issue with HLB sorbent was that an important amount of interfering compounds was simultaneously extracted according to the relatively low selectivity obtained with a single extraction procedure. An investigation of blank matrices was considered (Fig. 6) and non-spiked urine were extracted and analyzed in scan mode (100-500 *m/z*, corresponding to the detection window of expected analytes). The presence of interfering masses corresponding to endogenous compounds was observed in the detection window, which hampered a precise and accurate determination of analytes at low concentrations.

Double SPE

In order to obtain cleaner extracts, a sequential strategy involving two SPE steps was considered. The first one was achieved with a pure hydrophobic sorbent (C18) and the second one with a mixed-mode anion exchanger cartridge (OASIS® MAX). The free compounds were quantitatively retained by the C18 cartridge while the glucuronides were eluted during washing step with water. Compounds retained by hydrophobic interaction were eluted with 500 µL of acidic methanol (Fig. 7).

Subsequently, the washing eluate, containing the glucuronides, was loaded onto MAX cartridge after pH adjustment at a value of 9. Thanks to the anionic interaction, the second support enabled glucuronides extraction (Fig. 8) [34].

With this two-steps extraction procedure, it was possible to extract glucuronides in urine with a recovery of >80% and LOD down to 10 ng/mL were reached. A blank urine was also extracted and analyzed in scan mode (100-500 m/z) in order to evaluate the presence of remaining interfering compounds. The average abundance in the analytes detection window (6-8 min) using C18 cartridge (Fig. 7) was much lower

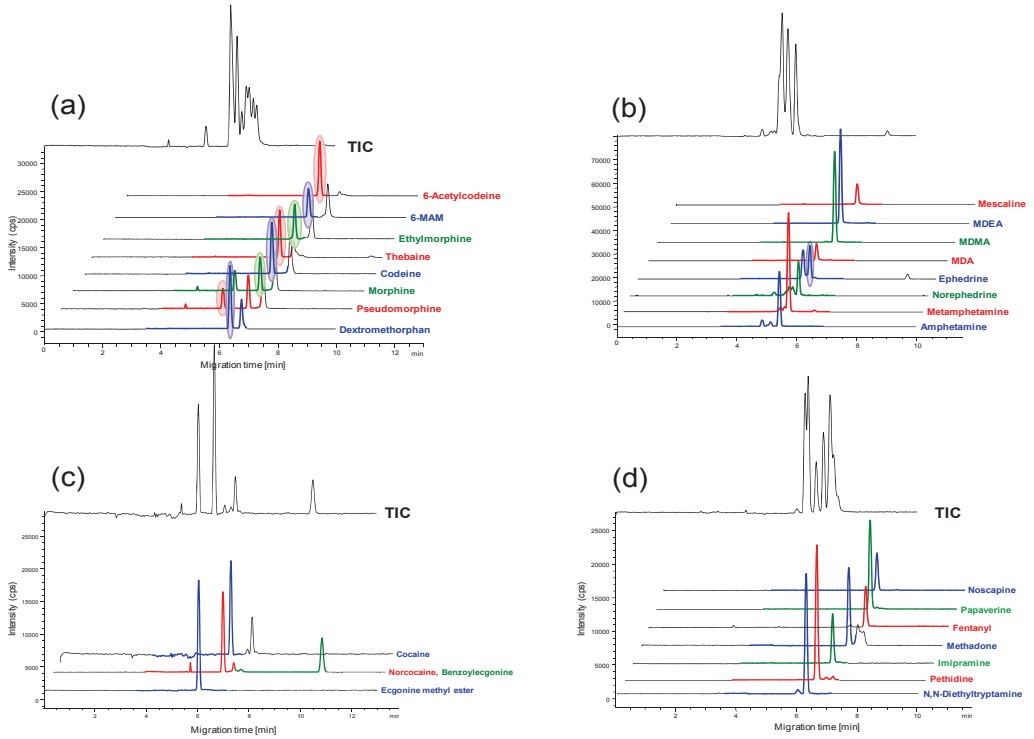

Fig. 5 CE-ESI-MS electropherograms obtained after solid-phase extraction (OASIS® HLB) of fortified urine containing (a) Morphine and analogs (100 ng/mL each); (b) Amphetamine and derivatives (100 ng/mL each); (c) Cocaine and metabolites (100 ng/mL each); (d) Other drugs (100 ng/mL each). FASI: 50 mbar for 3 s of water followed by 15 kV for 50 s of sample. Other experimental conditions as in Fig. 1.

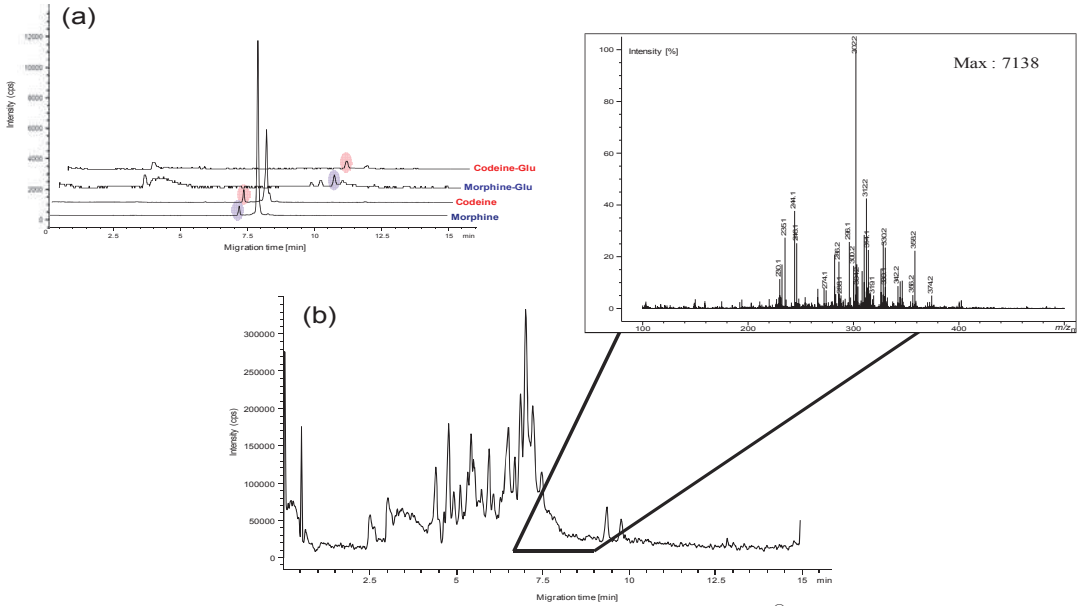

Fig. 6 CE-ESI-MS electropherograms obtained after solid-phase extraction (OASIS® HLB) of (a) fortified urine containing morphine, codeine, and their respective glucuronides (10 ng/mL each); (b) blank urine. FASI: 50 mbar for 3 s of water followed by 15 kV for 50 s of sample. Other experimental conditions as in Fig. 1, for (b) MS acquisition mode see text.

than with the hydrophilic-lipophilic cartridge (Fig. 6) (1563 counts vs 7138 counts, respectively).

The average abundance in the detection window (6-8 min) for the second extraction of the unretained polar

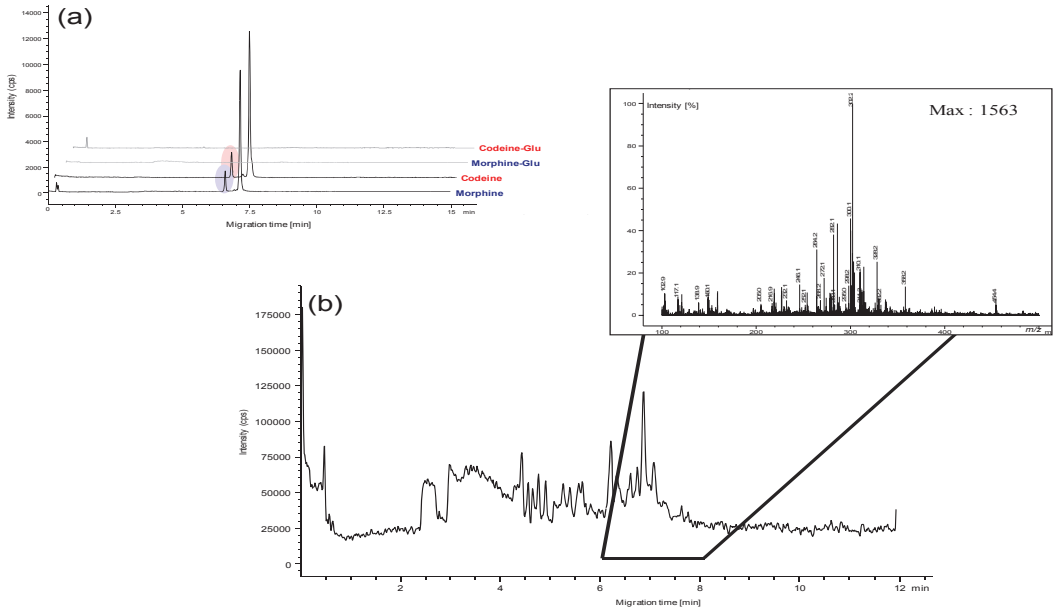

Fig. 7 CE-ESI-MS electropherograms obtained after solid-phase extraction with (C18) of (a) fortified urine containing morphine, codeine and their respective glucuronides (10 ng/mL each); (b) blank urine. FASI: 50 mbar for 3 s of water followed by 15 kV for 50 s of sample. Other experimental conditions as in Fig. 1, for (b) MS acquisition mode see text.

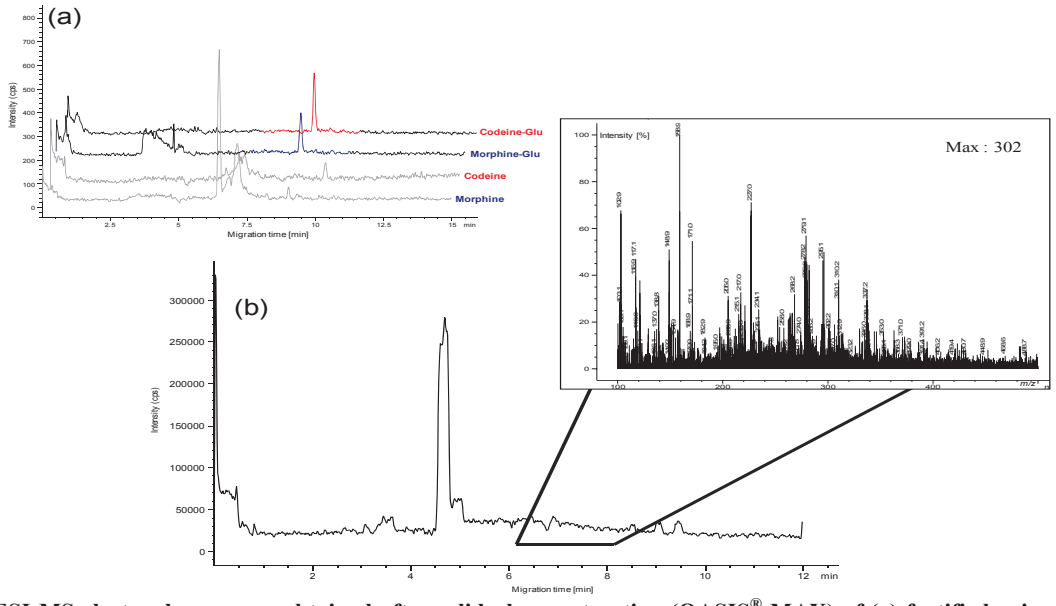

Fig. 8 CE-ESI-MS electropherograms obtained after solid-phase extraction (OASIS® MAX) of (a) fortified urine containing morphine, codeine and their respective glucuronides (10 ng/mL each); (b) blank urine. FASI: 50 mbar for 3 s of water followed by 15 kV for 50 s of sample. Other experimental conditions as in Fig. 1, for (b) MS acquisition mode see text.

fraction (i.e., glucuronides) was even lower (302 counts, Fig. 8), demonstrating the high selectivity afforded by the sequential SPE procedure. Therefore, the double extraction procedure with C18 and OASIS® MAX demonstrated lower matrix effects and allowed a clear revelation of glucuronides at a low concentration in urine (10 ng/mL each).

4. Conclusions

CE-MS is an attractive method for the analysis of drugs of abuse in urine. For rapidly gathering data from urine samples around the ppm level, diluted urine could

be directly injected into the CE-MS system. This sample pre-treatment was suitable for free analytes, as well as conjugated compounds (such as glucuronides) at a concentration higher than 1 µg/mL. In order to assess lower concentrations, compounds had to be extracted from urine. With LLE and electrokinetic injection, detection limits around 10 ng/mL were reached however this sample preparation procedure was not suitable for the simultaneous determination of apolar and very polar compounds such as glucuronides. With solid-phase extraction, detection limits were around 10 ng/mL, and suitable for free analytes and glucuronides, extracted with a two-steps SPE involving an initial extraction with a C18 sorbent followed by a second one with a mixed-mode anion exchanger cartridge.

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Appendix II.

Microextraction liquide-liquide dispersive combinée à l'électrophorèse capillaire et la spectrométrie de masse pour l'analyse de l'urine

I. Kohler, J. Schappler, T. Sierro, S. Rudaz, CJ'MAG 4 (2012) 3

Microextraction liquide-liquide dispersive combinée à l'électrophorèse capillaire et la spectrométrie de masse pour l'analyse de l'urine.

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Introduction

Durant ces dernières années, une grande attention a été portée sur l'utilisation de procédés chimiques présentant de faibles risques pour l'homme et l'environnement (approche dite de la chimie « verte »). Les 12 Principes de la Chimie Verte ont ainsi été édictés dans le but de pouvoir réduire ou éliminer l'utilisation de substances chimiques dangereuses [1]. Dans ce contexte, de récentes techniques analytiques ont été mises en évidence, comme par exemple la chromatographie liquide à ultra haute pression (UHPLC), la chromatographie en fluide supercritique (SFC) et l'électrophorèse capillaire (CE) dans lesquelles les quantités de modificateurs organiques utilisées sont fortement réduites. Cependant, lors de l'analyse de matrices complexes, la préparation de l'échantillon reste une étape critique en raison des grandes quantités de solvants organiques qui peuvent être requises dans le cas de l'extraction sur support solide (SPE) ou de l'extraction liquide-liquide (LLE) par exemple. Des techniques originales de préparation d'échantillon ont été mises au point ces dernières années telles que les techniques dites de microextraction où de très faibles quantités de solvants organiques sont utilisées [2,3]. Diverses approches miniaturisées de SPE ont déjà été proposées, comme la microextraction sur support solide (SPME) ou l'extraction sur barreau magnétique (SBSE). Afin de pallier la formation d'émulsions ou les quantités relativement importantes de solvants et d'échantillon utilisées en LLE, des techniques de microextraction liquide-liquide ont également été proposées, comme la microextraction en phase liquide sur fibre creuse (HF-LPME) ou la microextraction dispersive liquide-liquide (DLLME) [4,5].

La DLLME, introduite en 2006, est basée sur un système ternaire dans lequel un solvant dispersif permet la dispersion d'un solvant extractant dans l'échantillon [5-7]. Le solvant dispersif doit être complètement miscible avec l'échantillon aqueux et avec la phase extractante. Le solvant extractant doit être insoluble dans l'eau et avoir une densité supérieure à celle-ci, d'où le choix préférentiel de solvants halogénés. Un mélange des solvants extractant et dispersif est rapidement injecté dans l'échantillon, ce qui mène à la formation d'une multitude de petites gouttelettes et provoque une grande turbulence dans le liquide. La surface spécifique d'échange entre l'échantillon et les gouttelettes étant très importante, le temps d'extraction est fortement réduit grâce à un transfert d'analytes très rapide.

De nombreuses applications ont été développées avec les chromatographies gazeuse ou liquide ces dernières années sur diverses familles de composés. Cependant, jusqu'à aujourd'hui, peu d'études ont montré l'intérêt d'associer la DLLME à la CE comme technique d'analyse. Cette technique comporte de nombreux avantages comme une faible consommation de solvant, des temps d'analyse faibles, de hautes efficacités de

séparation ainsi que de faibles volumes d'échantillons requis pour l'injection, permettant ainsi des facteurs de préconcentration élevés.

Dans cette étude, la combinaison DLLME-CE a été évaluée pour le criblage de composés toxicologiques dans l'urine. En raison de la faible sensibilité de la détection UV conventionnellement utilisée en CE (faible chemin optique dû au diamètre du capillaire), cette dernière a été couplée à un analyseur de masse à temps de vol (TOF/MS) *via* une interface coaxiale avec ajout d'un liquide additionnel et une source de type électrospray (ESI). Dans le but d'obtenir les meilleurs rendements d'extraction, un plan d'expériences de type Plackett-Burmann a été appliqué sur deux composés modèles, le d-propoxyphène (DPX) et l'ecstasy (MDMA) pour déterminer les effets de 7 paramètres majeurs de la DLLME. Ce plan d'expériences a été réalisé en CE-UV afin de s'affranchir d'une éventuelle altération de signal observable en CE-ESI-TOF/MS. Des investigations complémentaires ont permis de choisir les valeurs optimales. Les performances de la méthode DLLME-CE-TOF/MS ont été évaluées puis la méthode appliquée à des cas toxicologiques.

Matériel et méthode

L'électrolyte de séparation (BGE) est constitué d'un tampon formate d'ammonium à pH 2.5, de concentration 20 mM. Les expériences sont réalisées sur des appareils CE et TOF d'Agilent Technologies (Waldbronn, Allemagne et Santa Clara, USA) avec un capillaire en silice fondue d'une longueur totale de 80 cm et de 50 µm de diamètre interne. Les échantillons sont injectés hydrodynamiquement à 50 mbar pendant 25 s (1.7% de la longueur totale du capillaire) et la séparation est réalisée à 25°C avec une tension de 30 kV et une rampe de voltage initial de 18 s (1667 V/s). Le liquide additionnel est composé d'isopropanol/eau/acide formique (50:50:0.5, *v/v/v*) infusé à un débit de 3 µL/min. La tension de fragmentation est fixée à 150 V, la tension ESI à +4500 V et la fréquence d'acquisition à 2.5 spectres/sec. Les spectres de masses obtenus sont automatiquement calibrés durant l'acquisition.

Les performances de la méthode DLLME-CE-TOF/MS sont évaluées selon une méthode développée par Matuszewski *et al*. [8] et complétée par Marchi *et al*. [9], et sont exprimées par l'efficacité du processus (PE), l'effet matrice (ME), le recouvrement d'extraction (RE) et le rendement d'extraction (EY) (Figure 1).

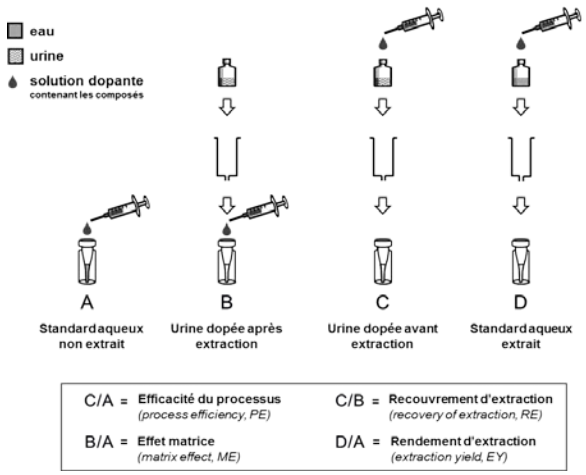

Figure 1 : mesure de l'efficacité du processus, de l'effet matrice, du recouvrement d'extraction et du rendement d'extraction. Adapté de [9].

Résultats

Les composés toxicologiques étudiés (*i.e.* amphétamines et dérivés, cocaïne et métabolites, opioïdes, composés pharmaceutiques, etc.) sont tous des molécules basiques présentant des pKa entre 6 et 10.5. Un pH d'échantillon supérieur à 11.5 a donc été choisi pour assurer un rendement d'extraction élevé des composés sous leur

forme neutre. L'urine étant préférentiellement acide, 1 mL de NaOH 1 M a été ajouté aux 4 mL d'urine de départ préalablement centrifugée puis filtrée.

Sélection des solvants

Le solvant d'extraction doit être plus dense que l'eau, avoir une bonne capacité d'extraction et une faible miscibilité avec l'eau mais aussi être soluble dans le solvant dispersif. Ce dernier doit être à la fois miscible dans l'eau et dans le solvant extractant. Parmi l'ensemble des solvants testés, le dichlorométhane et l'isopropanol ont été respectivement choisis comme solvant extractant et dispersif.

Criblage des paramètres DLLME

Sept paramètres ont été retenus comme pouvant influencer significativement la procédure : le volume de NaOH 1M utilisé pour l'ajustement du pH (X_1), le volume de mélange de solvants (X_2), le rapport de solvant extractant et solvant dispersif (X_3), la présence d'agitation (X_4), le temps d'extraction (X_5), la vitesse (X_6) et la durée (X_7) de centrifugation. Un plan d'expériences de type Plackett-Burmann a été utilisé pour le criblage de ces sept facteurs. L'efficacité du processus (PE, voir Figure 1) a été choisie comme réponse analytique. Le domaine investigué est présenté dans le tableau 1.

Tableau 1 : choix des bornes limites inférieures (-1) et supérieures (+1) du domaine investigué

Facteurs	Bornes		
	- 1	0	+ 1
X_1 Volume de NaOH 1M [mL]	0.4	1	2
X_2 Volume de mélange de solvants [mL]	1.0	1.5	2.0
X_3 $V_{extractant}/V_{dispersif}$ [%]	20	25	30
X_4 Agitation (O/N)	N	O	O
X_5 Temps d'extraction [min]	0	5	10
X_6 Vitesse de centrifugation [rpm]	4'000	7'000	10'000
X_7 Durée de centrifugation [min]	3	5	10

Les effets principaux sont exprimés par les valeurs de coefficient et de probabilité de chaque facteur (tableau 2). Les signes du coefficient indiquent l'effet du facteur (positif ou négatif). Une valeur de probabilité (p) inférieure à |5%| représente un effet significatif du facteur sur la réponse (PE). Une valeur entre |5%| et |10%| est considérée comme pouvant présenter un effet relatif sur la réponse. Une probabilité supérieure à |10%| indique que le facteur correspondant au coefficient n'a pas d'influence sur la réponse mesurée.

Tableau 2 : valeurs des coefficients et probabilités pour les sept facteurs criblés et les deux composés étudiés.

Facteurs	MDMA		DPX	
	Coefficient	Probabilité [%]	Coefficient	Probabilité [%]
X_1 Volume de NaOH 1M [mL]	+0.021	+22.2	+0.002	+91.4
X_2 Volume de mélange de solvants [mL]	+0.212	+2.4	+0.182	+4.4
X_3 $V_{extractant}/V_{dispersif}$ [%]	+0.021	+22.6	+0.023	+31.8
X_4 Agitation (O/N)	-0.046	-10.6	-0.049	-16.1
X_5 Temps d'extraction [min]	-0.156	-3.2	-0.124	-6.4
X_6 Vitesse de centrifugation [rpm]	+0.091	+5.5	+0.102	+7.8
X_7 Durée de centrifugation [min]	+0.092	+5.4	+0.069	+11.6

Concernant la MDMA, deux facteurs ont montré un effet significatif (p<|5%|) sur la réponse analytique: le volume de solvants (X_2) et le temps d'extraction (X_5) avec un effet positif pour le volume de solvants et négatif pour le temps d'extraction. Le volume de mélange de solvants (X_2) a été sélectionné à 2 mL pour un volume initial de 4 mL d'urine. Ce volume est particulièrement faible et correspond à moins de 500 µL de solvant organique par mL d'urine. Ceci est plus faible que le volume requis dans le cas d'une LLE conventionnelle. Le temps d'extraction (X_5) a été fixé à 0 min. La diminution du PE observée lors de l'augmentation du temps d'extraction peut être due à une rupture de l'émulsion formée par les trois phases. Ce temps d'extraction réduit à zéro est un des grands avantages de la DLLME et confirme un équilibre immédiatement atteint grâce à l'obtention d'une grande surface spécifique lors de la dispersion du solvant extractant.

La présence d'agitation (X_4), la vitesse (X_6) et la durée de centrifugation (X_7) ont montré un effet relatif (|5%|<p<|10%|). Aucune agitation (X_4) n'est donc requise selon le plan d'expériences, ce qui a été confirmé par les investigations complémentaires. Les paramètres de centrifugation (X_6 et X_7) qui affectent positivement l'extraction ont été fixés à 10'000 rpm pendant 5 min, offrant le meilleur compromis entre une séparation de phase suffisante, un bon PE et un temps d'extraction réduit.

Les autres facteurs (X_1 et X_3) n'ont pas d'effet significatif sur la réponse analytique. Le mélange de solvant organique contient donc 30% de dichlorométhane pour 70% d'isopropanol et le volume de NaOH ajouté à l'échantillon avant l'extraction a été fixé à 1 mL.

Le DPX montre les mêmes tendances pour les facteurs étudiés (Tableau 2).

Performances de la méthode DLLME-CE-TOF/MS développée

Comme une relativement grande quantité (jusqu'à plusieurs mL) d'urine peut être prélevée facilement, plusieurs volumes d'échantillons ont été testés avec la méthode d'extraction développée afin d'augmenter le facteur de préconcentration. Le meilleur compromis est observé avec 4 mL d'urine pour 2 mL de mélange de solvants. Le volume de reconstitution a également été évalué. En CE, bien que quelques nL soient injectés, les contraintes instrumentales requièrent un minimum de 10 µL dans le flacon d'injection. 30 µL ont donc été choisis comme volume de reconstitution pour assurer une injection répétable.

Ainsi, avec 4 mL d'urine avant extraction et 30 µL d'échantillon reconstitué, le facteur de préconcentration est égal à 133. Ce facteur élevé (assumant un PE théorique de 100%) augmente fortement la sensibilité de la méthode requise lors de criblages toxicologiques d'échantillons urinaires.

Les performances de la méthode DLLME-CE-TOF/MS ont ensuite été évaluées en termes d'effet matrice (ME), de recouvrement d'extraction (RE), de rendement d'extraction (EY) et d'efficacité du processus (PE). Ces quatre paramètres ont été calculés pour un ensemble de composés toxicologiques représentatifs de différentes classes chimiques courantes en toxicologie. Les résultats sont présentés dans le Tableau 3.

Tableau 3 : performances mesurées avec la méthode DLLME-CE-TOF/MS en termes d'effet matrice (ME), de rendement d'extraction (EY), de recouvrement d'extraction (RE) et d'efficacité du processus (PE).

	ME [%]	EY [%]	RE [%]	PE [%]
D-methorphane	44	75	107	47
D-PX	40	68	104	42
MTD	35	74	90	32
Morphine	98	0	0	0
Amphetamine	100	101	76	76
MDMA	90	85	75	68
Ephedrine	92	67	61	57
Codéine	95	88	74	70

Le PE est une combinaison du RE et de ME, et représente la performance absolue du processus analytique global. Les PE obtenus étaient satisfaisants (> 50%) pour la majorité des composés ne souffrant pas d'effet matrice ou de faible rendements d'extraction.

L'effet matrice (ME) peut être observé lors d'analyses CE-MS de matrices complexes, principalement avec une source de type ESI. Les altérations de signal (suppression ou augmentation de signal) sont dues à la co-migration de composés endogènes avec les analytes d'intérêt. Une préparation d'échantillons permet ainsi de diminuer la présence de ces composés [8,9]. Comme

indiqué dans le Tableau 3, un effet matrice important a été mesuré pour la méthadone (MTD), le DPX et le D-méthorphane avec des ME entre 35 et 44 %.

Le rendement d'extraction (EY) représente la performance de la préparation d'échantillon sur standards aqueux. Les composés ont présenté de bons rendements, hormis pour la morphine non extraite à cause d'une fonction acide totalement ionisée au pH de l'extraction.

Le recouvrement d'extraction (RE) exprime l'EY lors de l'extraction de la matrice. Habituellement, les composés endogènes présents dans l'échantillon ont un effet négatif sur l'extraction (diminution du RE par rapport à EY). Cependant, dans le cas d'extraction de l'urine, le RE peut être augmenté de par l'effet de sel inhérent à la matrice urinaire [10]. Cet effet de sel est observé pour le DPX, la MTD et le D-méthorphane (voir Tableau 3).

Application à des échantillons réels

Une urine blanche a été renforcée avec environ 30 composés toxicologiques et leurs métabolites de phase I principaux à 10 ng/mL. Cette urine a été extraite et analysée avec la méthode DLLME-CE-TOF/MS développée. Les électrophérogrammes obtenus sont présentés sur la Figure 2. Tous les composés ont été détectés. Les limites de détection (LOD) obtenues par cette méthode sont très bonnes puisqu'elles atteignent le *sub*-ng/mL pour certains des composés détectés.

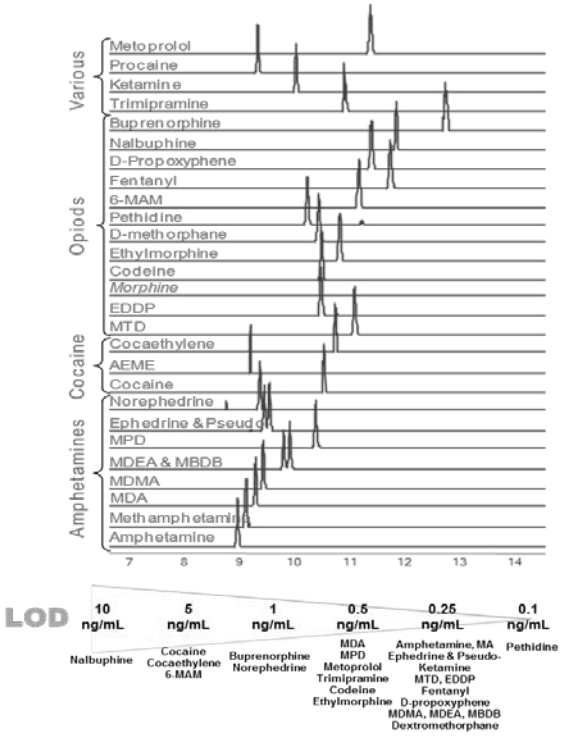

Figure 2 : électrophérogrammes obtenus avec la méthode DLLME-CE-TOF/MS et limites de détection (LOD) des composés étudiés.

Deux urines de toxicomanes ont été fournies par le Centre Universitaire Romand de Médecine Légale et du Laboratoire de Chimie Clinique des Hôpitaux Universitaires de Genève et ont été extraites puis analysées. Les électrophérogrammes obtenus se trouvent en Figure 3. Les composés ont été identifiés par comparaison des temps de migration avec des standards et par leur masse exacte. Le premier échantillon (Fig. 3a) contient de la cocaine et deux de ses métabolites, le cocaéthylène et l'anhydroecgonine méthylester (AEME). L'information apportée par la détection des métabolites est importante, puisque le cocaéthylène est observé lors d'une consommation concomitante de cocaine et d'éthanol, tandis que l'AEME se détecte lorsque la cocaine est consommée sous forme fumée (*crack*). Dans le second échantillon (Fig. 3b), l'amphétamine, l'ecstasy (MDMA) et la MDA ont été détectées. La détection de la MDA confirme la consommation d'ecstasy

puisque la MDA est un métabolite actif obtenu après *N*-méthylation de la MDMA.

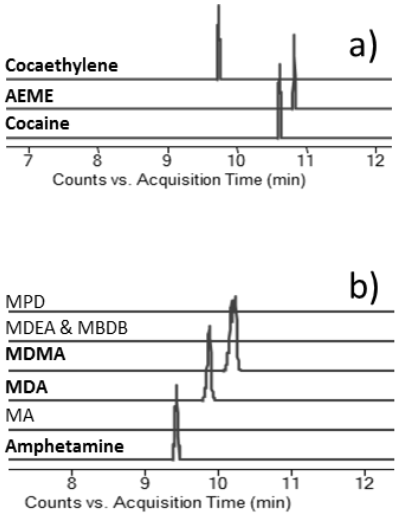

Figure 3 : électrophérogrammes obtenus pour des cas réels. a) cocaine et métabolites, b) amphétamines.

Conclusion

La combinaison de la DLLME et du couplage CE-ESI-TOF/MS a été évaluée pour le criblage toxicologique de composés basiques dans des échantillons urinaires. Sept facteurs de la procédure de microextraction ont été criblés à l'aide d'un plan d'expériences de type Plackett-Burmann, en configuration CE-UV avant des investigations complémentaires sur les paramètres significatifs pour augmenter l'extraction des composés d'intérêt. Les performances de la méthode développée ont été mesurées par plusieurs réponses liées à la qualité de l'extraction, mettant en évidence des différences de comportement selon les classes de molécules.

Un faible volume de solvants est utile pour réaliser l'extraction puisque moins de 500 µL de solvants sont requis pour chaque mL d'urine. Le facteur de préconcentration obtenu avec cette méthode est supérieur à 75 et peut atteindre 100.

La sensibilité élevée de la méthode développée a permis la détection d'environ 30 composés toxicologiques et pharmaceutiques dans l'urine avec des limites de détection pouvant atteindre le domaine du *sub*-ng/mL. Cette méthode a été appliquée à des cas réels avec détection des composés toxicologiques consommés et de certains de leurs métabolites de phase I[11].

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Appendix III.

Single-run separation of closely related cationic and anionic compounds by CE-ESI-MS: application to the simultaneous analysis of melamine and its analogs in milk

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Single-Run Separation of Closely Related Cationic and Anionic Compounds by CE-ESI-MS: Application to the Simultaneous Analysis of Melamine and its Analogs in Milk

Isabelle Kohler[ab], Emmanuelle Cognard[bc], Ivano Marchi[bc], Didier Ortelli[bc], Patrick Edder[bc], Jean-Luc Veuthey[ab], Serge Rudaz[ab], and Julie Schappler*[ab]

Abstract: In recent years, two adulteration incidents concerning the addition of melamine, a nitrogen-rich industrial small polar compound, to pet food and infant formula products have occurred in China. These issues prompted laboratories to develop methods for the analysis of melamine and related compounds in a wide variety of food products and ingredients. In this context, a CE-ESI-MS method was developed to simultaneously analyze melamine and its related products (ammeline, ammelide and cyanuric acid) that possess close physico-chemical properties. This method allows the simultaneous analysis of both cations and anions in a single run, using CE to divide the run into two time segments in normal polarity mode. For this purpose, ESI polarity was switched once during the run, increasing sensitivity and data quality. The method was applied to spiked powdered milk and melamine-contaminated powdered milk, with two sample preparation procedures.

Keywords: CE-MS · Cyanuric acid · Food analysis · Melamine · Single run

1. Introduction

Melamine (MEL), 2,4,6-triamino-1,3,5-triazine (Fig. 1), is an emerging contaminant that has been illegally added to dairy products such as milk, infant formula, and pet food. Adding MEL to food increases its nitrogen content, artificially boosting the protein level, especially when indirect protein assays based on total nitrogen are employed (Kjeldahl method).[1,2] Commercially synthesized MEL can contain structural by-products, such as ammeline (AMLN), ammelide (AMLD), and cyanuric acid (CYA, Fig. 1). MEL is not metabolized and is rapidly excreted in urine. Long-term exposure can reduce fertility and result in fetal toxicity.[2] In addition, MEL and related triazines (particularly CYA) can form high molecular weight complexes through hydrogen bonding, leading to formation of insoluble crystals in kidneys and resulting in kidney stones and renal failure.[2–4] Based on studies on rats, a tolerable daily intake (TDI) of 0.2 ppm body weight was established for MEL and extrapolated to 1.5 ppm body weight for CYA by the World Health Organization. Therefore, many countries have introduced limits for MEL in food products that provide a sufficient margin of safety related to the TDI, *i.e.* 1 ppm in infant formula and 2.5 ppm in other food.

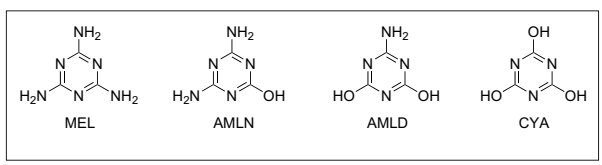

Fig. 1. Chemical structure of melamine and its related products.

Until now, the analytical methods developed to analyze MEL in food[5] were enzyme-linked immunosorbent assay,[6] enzyme immunoassay,[7] surface-enhanced Raman spectroscopy,[8] GC-MS,[9–11] and LC-MS.[12–20] Some of these methods also meet the growing need to detect MEL by-products because of their deleterious effects on health.[21–24] CE methods were also developed to analyze MEL with UV or MS detection.[25–34] Capillary electrophoresis (CE) is a powerful separation technique for polar compounds with several advantages including high separation efficiency, low sample and solvent consumption, short analysis time, and simple instrumentation. UV-vis spectrophotometry is probably the most widely used detection technique due to the simplicity of the on-line configuration. However, this technique suffers from a lack of sensitivity because of the narrow optical path length allowed by the internal diameter of the capillary. Consequently, high analyte concentrations, which are rarely available in bioanalysis and food matrices, are required. To circumvent this lack of sensitivity, CE can be on-line coupled with mass spectrometry (MS). The latter is the preferred detection method for these applications due to its quasi-universality, high sensitivity, high selectivity, and ability to identify compounds in complex mixtures *via* mass-to-charge ratio (*m/z*).

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Electrospray ionization (ESI) is the most widespread ionization source used for hyphenating CE and MS,[35] and two main configurations can be distinguished, either with or without the addition of a make-up liquid. The first approach is the most common, particularly with a coaxial sheath-flow interface. In this configuration, the sheath liquid mixes with the CE effluent at the tip of the separation capillary, providing electrical contact at the outlet end of the capillary, and the appropriate flow rate and solvent conditions for ionization and evaporation of the analytes.[36–38]

The main limitation of CE-ESI-MS concerns the use of volatile electrophoretic buffers (*e.g.* acetate, formate, carbonate, ammonium), and/or volatile selectivity modifiers to avoid the putative contamination of the ESI source.[39] Conventionally, an acidic BGE, a sheath liquid containing formic or acetic acid, and ESI in the positive mode (ESI+) are used for cation analysis, while basic BGE and sheath liquid with ammonium are used for anion analysis in negative ESI mode (ESI-). Thus, simultaneous analysis of both cations and anions by CE-ESI-MS in a single run is not directly achievable and the capillary, the BGE, and the sheath liquid have to be adapted between applications. Several attempts were made by CE-UV to overcome this issue, using techniques such as complexing cations with EDTA in reversed polarity mode,[40] addition of poly(diallyldimethylammonium chloride) to the BGE,[41] use of a coated capillary,[42] and a dual-opposite injection approach, which consists of simultaneous injection from both sides of the capillary with the detector placed approximately in the center.[43–46] However, these techniques can hardly be implemented when MS is used for detection. Therefore, it remains challenging to simultaneously analyze cations and anions by CE-ESI-MS. In the present paper, a CE-ESI-MS method is presented to allow the simultaneous detection of MEL, AMLN, AMLD, and CYA with adequate analytical conditions in a single run in spiked and contaminated powdered milk.

Table 1. Physico-chemical, electrophoretic, and MS properties of MEL and its related products.

	MEL	AMLN	AMLD	CYA
Physico-chemical properties				
Molecular weight [Da]	126.07	127.05	128.03	129.02
Acidic $pK_a \pm IC_{95\%}$ [a]	-	9.55 ± 0.18	6.97 ± 0.09	6.89 ± 0.06 11.40 ± 0.08
Basic $pK_a \pm IC_{95\%}$ [a]	5.12 ± 0.04	4.21 ± 0.18	1.58 ± 0.11	-
Electrophoretic properties				
Ionization at pH 5.2	45% (cation)	9% (cation)	2% (anion)	2% (anion)
Mass spectrometry properties				
Detected ions	$[M+H]^+$	$[M+H]^+$	$[M-H]^-$ $[2M-H]^-$	$[M-H]^-$
m/z	127	128	127 255	128
Fragmentor voltage [V]	140	140	90	90

[ab] pK_a values experimentally determined according to Geiser *et al.*[49]

2. Material and Methods

2.1 *Chemicals*

Acetic acid, ammonia, hydrochloric acid, ammonium acetate, sodium hydroxide, ethanol (EtOH), formamide, N-methylformamide (NMF), and N,N-dimethylformamide (DMF) of analytical reagent grade were obtained from Fluka (Buchs, Switzerland). Acetonitrile (ACN) and methanol (MeOH) of analytical reagent grade were obtained from Panreac Quimica SA (Castella del Vallès, Spain), analytical grade isopropanol (iprOH) from Acros Organics (Geel, Belgium), and formic acid from Biosolve (Valkenswaard, Netherlands). Ultrapure water was supplied by a Milli-Q RG purification unit from Millipore (Bedford, MA, USA).

MEL, AMLN (atrazin-desethyl-desisopropyl-2-hydroxy), CYA were purchased from Sigma-Aldrich (Seelze, Germany), and AMLD was obtained from TCI Europe (Zwijndrecht, Belgium). They possess close physico-chemical properties (Fig. 1 and Table 1).

2.2 *Background Electrolyte and Sample Preparation*

2.2.1 *BGE*

The BGE consisted of a 25 mM (ionic strength) ammonium acetate buffer set at pH 5.2. For confirmatory analysis of AMLD and CYA, a 25 mM (ionic strength) ammonium acetate buffer set at pH 9.0 was used. The pH values were measured with a SevenMulti pH meter (Mettler-Toledo, Schwerzenbach, Switzerland).

2.2.2 *Sample Preparation*

Stock standard solutions of the analytes were prepared by dissolving each reference compound in MeOH to obtain a concentration of 1 mg/mL (1000 ppm) for MEL and CYA, and 0.05 mg/mL (50 ppm) for AMLN and AMLD. Standard solutions at desired concentrations were prepared daily by diluting stock solutions in water.

The developed CE-ESI-MS method was tested with the following real samples: i) delipidated blank powdered milk Rapilait purchased from a local retailer store (Migros, Geneva, Switzerland), reconstituted as instructed on the packaging and spiked with MEL, AMLN, AMLD at 5 ppm, and CYA at 1 ppm and ii) Chinese positive powdered milk provided from an interlaboratory study performed by the Food Authority Control of Geneva (Geneva, Switzerland) and reconstituted as required for this study.

A sample preparation procedure based on solid-phase extraction (SPE) was chosen to allow for the extraction of all compounds. Two different materials were studied: i) a new Strata Melamine cartridge from Phenomenex (Torrance, USA) which theoretically enables the simultaneous extraction of MEL and CYA and ii) a mixed-mode cation exchanger (MCX) Oasis cartridge from Waters (Milford, USA), used as the reference sample preparation procedure.[12,47]

2.2.2.1 *SPE with Strata Melamine*

Strictly following the manufacturer protocol, 100 µL of 0.2 M HCl and 3 mL of ACN were added to 1 mL of reconstituted milk, vortexed, and centrifuged at 6000 rpm for 10 min. The supernatant was loaded onto the cartridge (200 mg sorbent, 3 mL), previously conditioned with 3 mL of MeOH and 3 mL of water at 1 mL/min. The first washing step was performed with 1 mL of ACN-water (50:50, v/v) and the second with 500 µL of MeOH-water (50:50, v/v). The sorbent was dried for 2 min at 10 psi. The compounds were eluted with 500 µL of MeOH and 1 mL of MeOH-ammonia (95:5, v/v). The elution fraction was evaporated to dryness under a gentle steam of nitrogen and reconstituted with 1 mL ACN-20 mM ammonium acetate (95:5, v/v), then injected into the CE-MS system.

2.2.2.2 *SPE with MCX*

Ten milliters of 0.12 M HCl were added to 5 g of reconstituted milk, and the mixture was vortexed for 45 s and centrifuged at 4000 rpm for 5 min. The supernatant was loaded onto the cartridge (150 mg sorbent), which was previously conditioned with 5 mL MeOH and 5 mL water at 1 mL/min.

The washing step was done with 5 mL 0.1 M HCl and 2 mL MeOH. Compounds were eluted with 5 mL ACN-ammonia (95:5, v/v). One mL of the elution fraction was evaporated to dryness under a gentle steam of nitrogen and reconstituted with 1 mL ACN-20 mM ammonium acetate (95:5, v/v), then injected into the CE-MS system.

2.3 *Instrumentation*

2.3.1 *Capillary Electrophoresis*

CE experiments were performed with an HP ^{3D}CE system from Agilent (Waldbronn, Germany) equipped with an on-capillary diode array detector, an autosampler, and a power supply able to deliver up to 30 kV. Separation was performed using a fused-silica capillary (BGB Analytik AG, Böckten, Switzerland) with a total length of 80 cm and an internal diameter of 50 µm. Before its first use, the capillary was rinsed sequentially at 2 bar with MeOH (6 min), water (3 min), 0.1 M NaOH (6 min), water (3 min), 0.1 M HCl (6 min), water (3 min), and BGE (6 min). The capillary was conditioned daily with MeOH and water at 2 bar (3 min each). Prior to each sample injection, the capillary was rinsed at 2 bar with fresh BGE (2 min). When not in use, the capillary was rinsed with water and then dry-stored. Samples were kept at ambient temperature in the autosampler and injected hydrodynamically at 50 mbar for 25 s (corresponding to 1.7% of the capillary length). Experiments were carried out in positive polarity mode (anode at the inlet and cathode at the outlet). A constant voltage of 30 kV with an initial ramping of 1667 V/s (18 s) was applied, and the capillary was thermostated at 25 °C.

2.3.2 *Mass Spectrometry*

MS detection was performed with a single quadrupole Agilent Series 1100 MSD (Palo Alto, CA, USA). The electrospray ionization was carried out sequentially in positive and negative modes with time segments. A CE-MS adapter kit interface from Agilent was used to interface the HP ^{3D}CE instrument with the mass spectrometer. The composition of the coaxial sheath liquid was a mixture of iprOH-water-ammonia (50:50:2, v/v) containing 25 mM acetic acid. The sheath liquid was delivered by a Jasco PU-980 HPLC Pump (Omnilab, Mettmenstetten, Switzerland) at 3 µL/min. The ESI capillary voltage was set at 4300 V in the positive mode and 3400 V in the negative mode. The nebulizing pressure and drying gas flow rate were set at 4 psi and 4 L/min, respectively, while the drying gas temperature was set at 250 °C. These values were selected according to Geiser *et al.*[48] In preliminary experiments, MEL, AMLN, AMLD were infused at 50 ppm each, and CYA at 250 ppm in the BGE to determine their optimal fragmentor values, resulting in the highest intensity for the most abundant ions (Table 1).

2.4 *pKa Determination*

Given the numerous predictive pK_a values found in the literature for the four compounds, experimental pK_a values were determined by CE-UV using a dynamic coating procedure.[49] Knowing accurate pK_a values allowed the best pH value for BGE, at which all the compounds were partially ionized, to be selected. Because the pK_a value depends on BGE ionic strength and temperature, the values used were calculated for 25 mM and 25 °C.

2.5 *Software*

BGEs were prepared with the help of PHoEBuS software (version 1.3, Analis, Namur, Belgium). CE ChemStation (version B.01.03, Agilent, Waldbronn, Germany) was used for instrument control, data acquisition, and data handling. Prism software (version 4.0, GraphPad Software, San Diego, CA, USA) was used to determine pK_a values using experimental electrophoretic mobilities at different pH values.

3. Results and Discussion

3.1 *Development of Analytical Conditions*

All compounds present similar structures and therefore have close physicochemical properties (Fig. 1 and Table 1). Hence, the challenge was to find BGE conditions able to separate compounds according to their molecular size or ionization percentage. pK_a Values can aid in the choice of buffer pH to obtain the best selectivity. Predicted pK_a values were 5.6 ± 0.2 (basic) for MEL, 4.0 ± 0.2 (basic) and 6.2 ± 0.7 (acid) for AMLN, 1.5 ± 0.7 (basic) and 6.8 ± 0.7 (basic) for AMLD, and 5.2 ± 0.2 (acid) for CYA. Because theoretical pK_a values were not accurate, they were experimentally determined for the four compounds.

3.1.1 *pKa Determination and BGE Conditions*

Results obtained from experimental pK_a determination, as described by Geiser *et al.*,[49] are summarized in Table 1. The selection of a BGE that allowed electrophoretic separation between the four analytes at a pH value in which all compounds were ionized was then performed. Conventionally, MEL and AMLN (possessing basic pK_a values) can be separated as cations under acidic conditions (pH <6), whereas AMLD and CYA (possessing acidic pK_a values) can be analyzed as anions in neutral or basic conditions (pH >6).

Numerous BGEs were tested, including 25–100 mM ammonium acetate and ammonium formate buffers between pH 4.0–7.0, in steps of 0.25. The influence of organic solvent addition was also investigated because it might allow selectivity changes. Furthermore, the presence of an organic solvent may i) enhance peak efficiency, ii) be more easily evaporated for ESI-MS detection, and iii) increase sensitivity.[50] Six different organic modifiers were tested: MeOH, EtOH, ACN, formamide, NMF, and DMF, added from 10 to 50% (v/v) to the BGE. Pure non-aqueous conditions were also investigated but did not lead to separative improvement regardless of the nature of the organic solvent or its concentration in the BGE. In summary, all compounds were partially ionized in a 25 mM (ionic strength) ammonium acetate buffer set at pH 5.2 (Table 1). Under these conditions, MEL and AMLN were separated and migrated as cations before the EOF, whereas AMLD and CYA co-migrated as anions after the EOF (Fig. 2).

3.1.2 *Interface and MS Conditions*

To obtain a stable and repeatable spray in the ESI source, interface parameters, such as ESI voltage and sheath liquid composition, were evaluated. Applied voltages were set at 4300 V and 3400 V in ESI+ and ESI-, respectively, to allow good ionization with stable ESI currents. The sheath liquid was an iprOH-water mixture that afforded electrical connection to close the CE circuit and evaporation of CE effluent at the tip of the capillary.[37,48,51]

Conventionally, a small percentage of acid is added to the hydro-organic mixture for basic compounds ionization (*e.g.* formic acid, TFA) to assist analyte protonation, and a small amount of ammonia is added to increase deprotonation of acidic compounds. To analyze MEL and its by-products, a sheath liquid composed of a mixture of water-iprOH (50:50, v/v) with ammonia was investigated to allow simultaneous protonation of MEL and AMLN and deprotonation of AMLD and CYA. Ammonia has the ability to deprotonate acidic analytes while providing a proton to basic compounds, allowing all the analytes to be ionized.[52,53] Its concentration was varied to obtain a compromise between analyte sensitivity and spray stability, which was achieved at 2% (v/v). Acetic acid of 25 mM concentration was also added to the sheath liquid to stabilize CE and ESI currents and reduce baseline fluctuations in the positive and negative modes. Optimized fragmentor voltages, which resulted in the highest intensity for all compounds, were set at 140 V for MEL and AMLN, and 90 V for AMLD and CYA.

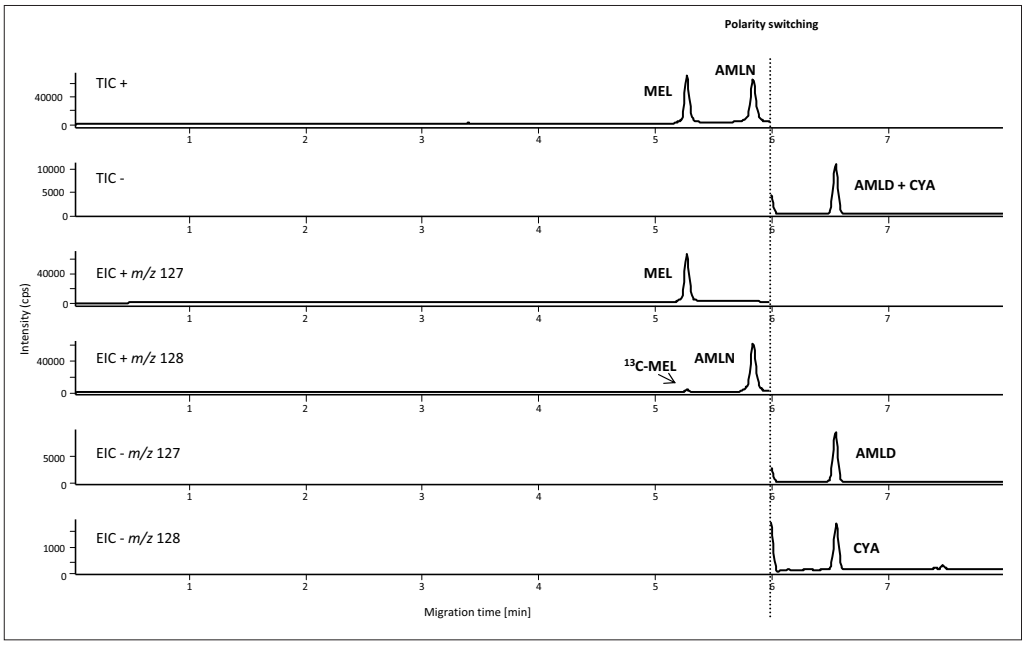

Fig. 2. CE-ESI-MS electropherograms obtained at pH 5.2 by injecting a mixture of MEL, AMLN, AMLD, and CYA at 25 ppm (each in water). ESI+ from 0 to 6.0 min, switch of polarity at 6.0 min (300 ms), and ESI- from 6.3 to 8 min. See text for experimental conditions.

3.1.3 *Acquisition Mode*

To achieve a simultaneous MS detection of cations and anions within a single run, ESI polarity can be switched throughout the entire run and simultaneous ESI+/- signals can be monitored. However, this might induce a loss in sensitivity attributable to the time necessary to perform polarity switching. Moreover, this could also imply a loss in data quality because the cycle time would greatly increase in simultaneous ESI+/- mode compared to single ESI mode, resulting in low acquisition rates.[53] Because it is important to maintain at least 15 acquisition points per peak for quantitative purpose, this could be barely possible with the narrow peak width obtained in CE (*ca*. 6 s). Using the unique feature of zone electrophoresis that allows separation between cationic compounds in the first run segment and anionic compounds in the second, the division of the run into two time segments for MS acquisition was considered. Consequently, ESI-MS responses were obtained in a single polarity mode (*i.e.* one recorded TIC trace, either ESI+ or ESI-), giving more sensitive results. From the beginning of the run to the polarity switch, a first window corresponding to the migration of cationic compounds was monitored in positive mode, and two ions were detected, *m/z* 127 and 128, corresponding to $[M+H]^+$ of MEL and AMLN, respectively. From the switch to the end of the run, a second window corresponding to the anionic species was monitored in negative mode and ions *m/z* 127 and 128 were detected, corresponding to $[M-H]^-$ of AMLD and CYA, respectively. The time of the polarity switch was determined each day by injecting an EOF marker prior to analyses. The marker was also injected every five runs to correct the time of the switch because of EOF migration time variability. It should be noted

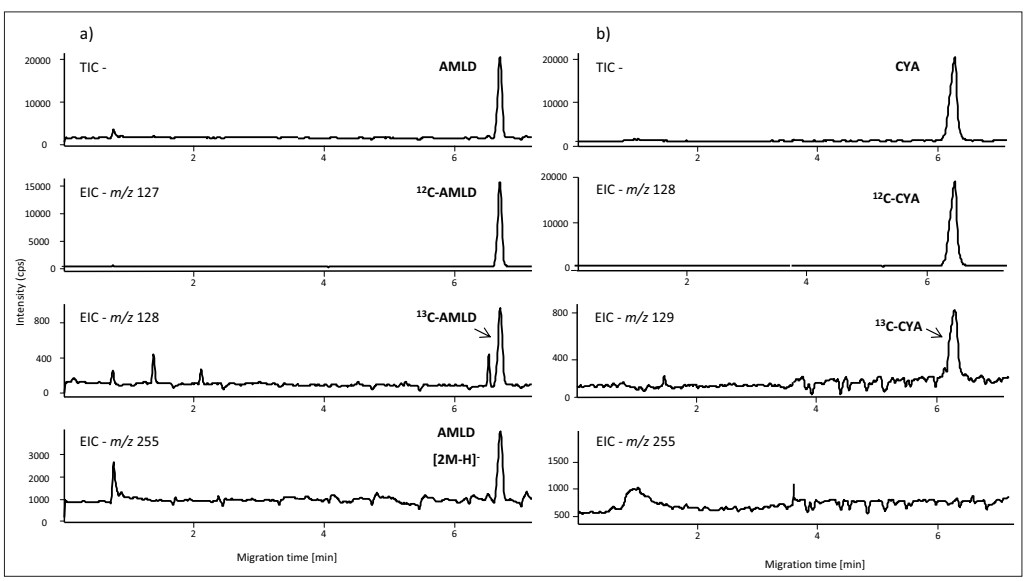

Fig. 3. a) CE-ESI-MS electropherograms obtained in ESI- at pH 5.2 by injecting AMLD at 25 ppm in water. b) CE-ESI-MS electropherograms obtained in ESI- at pH 5.2 by injecting CYA at 25 ppm in water. See text for experimental conditions.

that the switch between both polarities was achieved in 300 ms. Under these conditions, the limits of detection (LOD), corresponding to a signal-to-noise ratio (S/N) of 3, were 200 ppb for MEL, AMLD, CYA, and 500 ppb for AMLD.

An important issue concerned the limited spectral resolution of the single quadrupole mass spectrometer (*m/z* 0.7 FWHM) that did not permit straightforward MS discrimination due to the small *m/z* difference between the analytes. This was not critical for cations because the electrophoretic selectivity between MEL and AMLN allowed their physical separation despite the putative interference of MEL on AMLN MS-trace due to ^{13}C-isotopic contribution of MEL (Fig. 2). However, the resolution was insufficient for anions; AMLD and CYA were not separated by either CE or MS because of the ^{13}C-isotopic contribution of AMLD on CYA MS-trace. MS spectra of both compounds performed in scan mode between mass range *m/z* 100–1000 revealed the presence of the AMLD dimer form $[2M-H]^-$ at *m/z* 255. The latter was thus included as a confirmatory ion for AMLD identification (Fig. 3). When in doubt, other operating conditions could be performed with a BGE at basic pH in ESI- mode with the same sheath liquid composition. For example, a 25 mM (ionic strength) ammonium acetate BGE set at pH 9.0 allowed a complete electrophoretic separation of AMLD and CYA (Fig. 4).

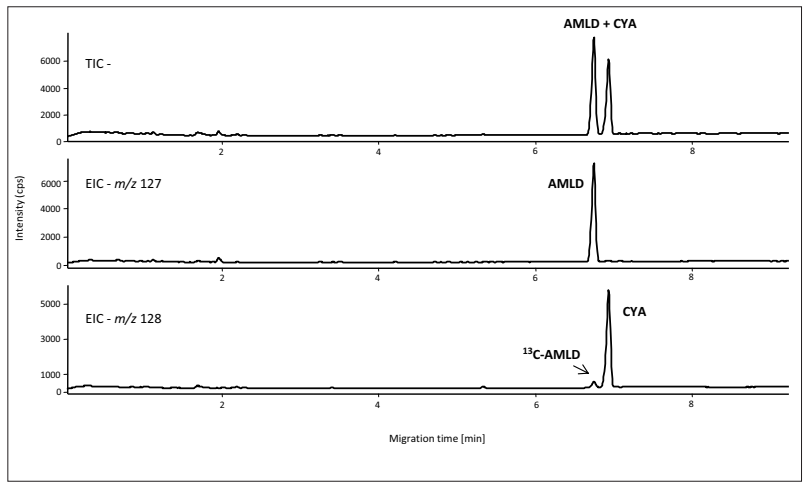

Fig. 4. CE-ESI-MS electropherograms obtained in ESI- at pH 9.0 by injecting a mixture of AMLD and CYA at 25 ppm (each in water). See text for experimental conditions.

3.2 *Application to Real Samples*

A new cartridge was introduced on the market that would allow the simultaneous extraction of both MEL and CYA via strong cationic exchange and hydrophilic lipophilic interactions, and was tested for MEL and its by-products. The protocol developed by the manufacturer was strictly followed for the extraction of Rapilait milk spiked with the four analogs. Results are shown in Fig. 5a and were not satisfactory in terms of peak shape for MEL and AMLN, whereas AMLD and CYA were absent on the electropherogram. To confirm the previous results, this material was evaluated with aqueous standard solutions

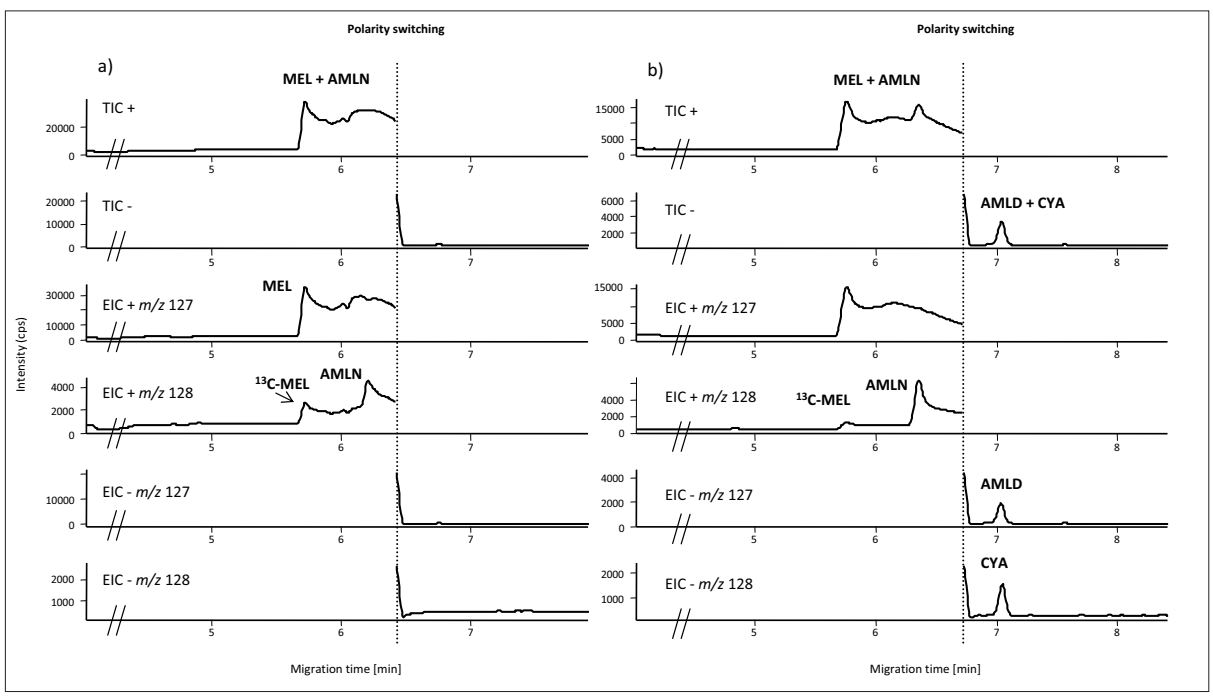

Fig. 5. a) CE-ESI-MS electropherograms obtained by injecting elution fraction after extraction with Strata Melamine cartridge of powdered Rapilait milk spiked with MEL, AMLN, AMLD at 5 ppm, and CYA at 1 ppm. ESI+ from 0 to 6.4 min, switch of polarity at 6.4 min (300 ms), and ESI- from 6.7 to 8.4 min. b) CE-ESI-MS electropherograms obtained by injecting elution fraction after extraction with Strata Melamine cartridge of standard solutions spiked with MEL, AMLN, AMLD, and CYA at 25 ppm. ESI+ from 0 to 6.7 min, switch of polarity at 6.7 min (300 ms), and ESI- from 7.0 to 8.7 min. See text for experimental conditions.

at 25 ppm. At this concentration, electropherograms obtained for MEL and AMLN were still not satisfactory (Fig. 5b), while AMLD and CYA were present. Therefore, this material seemed sufficient to extract high concentrations of AMLD and CYA, but did not give adequate results for MEL and AMLN, in either standard solutions or spiked milk. Although operating conditions were investigated, in terms of i) cartridge conditioning, ii) loading step (pH of the sample), iii) washing step (nature and proportion of solvent), iv) elution step (nature and volume of the solvent), and v) flow through the cartridge, no convincing or repeatable results were obtained, particularly for MEL and AMLN.

In a second set of experiments, Rapilait milk spiked with the four analogs was extracted with a strong cationic exchanger according to a protocol routinely used to extract only MEL from solid food product. The extracted sample was analyzed with the developed CE-ESI-MS method and the electropherograms are presented in Fig. 6a. MEL and AMLN were present on the electropherograms, while AMLD and CYA were absent, according to the selective retention mechanism of MCX material that could only retain cationic compounds, which were MEL and AMLN in this study. An S/N of 145 was obtained for MEL in spiked milk at 5 ppm. This result demonstrates the applicability of the developed CE-ESI-MS method for the analysis of contaminated samples, considering to the regulatory cut-offs of 1 ppm in infant formula and 2.5 ppm in other food products. Chinese reconstituted milk contaminated with MEL obtained from an interlaboratory study was also analyzed and showed presence of MEL on the electropherogram, while presence of AMLN was excluded (Fig. 6b). These results were confirmed by an LC-MS analysis performed at the Food Authority Control of Geneva (Geneva, Switzerland). AMLD and CYA, which were not extracted with this material due to the selective retention mechanism, could be extracted in a second step using mixed-mode anion exchanger (MAX) cartridges.[54]

4. Conclusions

The development of powerful analytical techniques in the context of food analysis is of prime importance. This was recently illustrated by dairy products adulteration with MEL, a nitrogen-rich compound that was illegally added to infant formulas, causing severe damage to the human urinary system. In this study, MEL and its by-products (AMLN, AMLD, and CYA) were analyzed within a single run with CE-ESI-MS. The method was applied to real samples, such as spiked blank powdered milk and contaminated powdered milk. With a 25 mM (ionic strength) ammonium acetate BGE set at pH 5.2, MEL and AMLN migrated as cations in the first part of the run, and AMLD and CYA as anions in the second part. The unique feature of CE permitted the division of the run into two time segments for MS detection, enhancing sensitivity and data quality. To assist the simultaneous protonation of MEL and AMLN and the deprotonation of AMLD and CYA in the ESI source, 2% ammonia was added to the sheath liquid composed of a mixture of water-iprOH (50:50, v/v). With these conditions, all compounds could be resolved either by CE or by MS, and LOD down to 200 ppb were reached. Two sample preparations were tested in spiked blank powdered milk and contaminated powdered milk. MCX cartridges allowed the extraction of MEL and AMLN, but not AMLD and CYA. A new material was also tested with a protocol developed by the manufacturer but peak shapes obtained for MEL and AMLN were not satisfactory, while AMLD and CYA could not be detected at a concentration lower than 10 ppm.

List of Abbreviations

ACN	Acetonitrile
AMLD	Ammelide

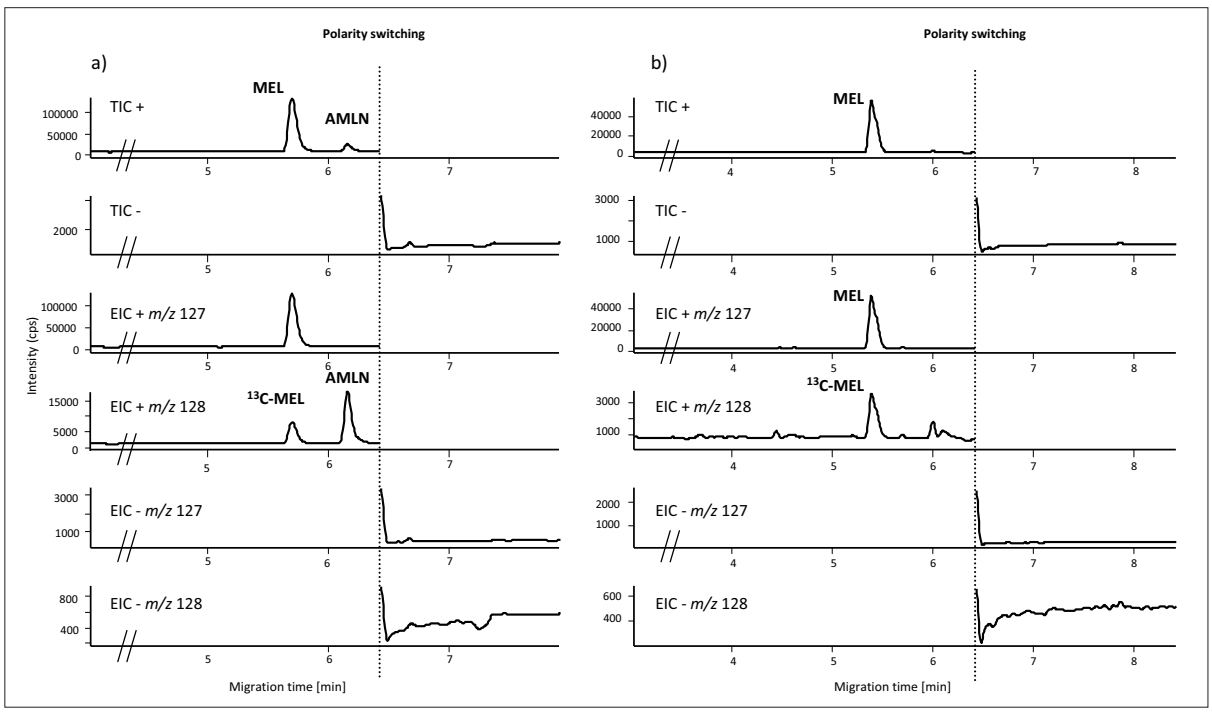

Fig. 6. a) CE-ESI-MS electropherograms obtained at pH 5.2 by injecting elution fraction after MCX extraction of powdered Rapilait milk spiked with MEL, AMLN, AMLD at 5 ppm, and CYA at 1 ppm. ESI+ from 0 to 6.4 min, switch of polarity at 6.4 min (300 ms), and ESI- from 6.7 to 8.4 min. b) CE-ESI-MS electropherograms obtained by injecting elution fraction after MCX extraction of contaminated powdered milk (interlaboratory study). ESI+ from 0 to 6.4 min, switch of polarity at 6.4 min (300 ms), and ESI- from 6.7 to 8 min. See text for experimental conditions.

AMLN	Ammeline
BGE	Background electrolyte
CYA	Cyanuric acid
DMF	N,N-dimethylformamide
EIC	Extracted ion current
ESI	Electrospray ionization
EtOH	Ethanol
FWHM	Full width at half maximum
iprOH	Isopropanol
LOD	Limit of detection
MEL	Melamine
MeOH	Methanol
m/z	Mass-to-charge ratio
NMF	N-methylformamide
SIM	Selected ion monitoring
SPE	Solid-phase extraction
TDI	Tolerable daily intake
TIC	Total ion current

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Appendix IV.

Compatibility of Agilent Jet Stream thermal gradient focusing technology with CE/MS

I. Kohler, J. Schappler, S. Rudaz, H.P. Zimmermann, C. Wenz, Agilent Technologies Technical Note, publication number 5990-9716EN (2012)

Compatibility of Agilent Jet Stream thermal gradient focusing technology with CE/MS

Technical Overview

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Abstract

Coupling capillary electrophoresis to mass spectrometers through the Agilent triple tube coaxial sheath-flow sprayer is a widely used configuration due to its simplicity, versatility, and robustness. This Technical Overview shows that with the Jet Stream source and the latest version of the CE/MS sprayer, this approach can now also be used in combination with the proprietary Agilent Jet Stream thermal gradient focusing technology. The sprayer worked as well on a mass spectrometer equipped with a conventional ESI source and showed, in comparison to the standard sprayer, a slightly increased performance. When the sprayer was used in combination with an Agilent Jet Stream source, equivalent or better results were obtained as compared to a conventional ESI source.

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Introduction

Achieving improvements in sensitivity and efficiency for electrospray ionization (ESI) has been a challenge over the past few decades, however, these improvements are important for multiple analytical applications. One recent innovation was the introduction of the thermal gradient focusing technology that was implemented in the Agilent Jet Stream source[1,2]. In this design, a superheated nitrogen sheath gas confines the nebulizer spray to more efficiently dry ions and concentrates them in front of the sampling orifice of the mass spectrometer. Due to improved ion generation and sampling efficiency, a 5 to 10-fold improved sensitivity can be obtained in comparison to conventional ESI in liquid chromatography coupled with mass spectrometric detection (LC/MS). The Agilent Jet Stream source is the standard source that is delivered with all Agilent mass spectrometry systems.

This Technical Overview demonstrates the compatibility of the Agilent Jet Stream thermal gradient focusing technology with capillary electrophoresis coupled with mass spectrometric detection (CE/MS). For use with the Agilent Jet Stream source, an updated triple tube coaxial sheath-flow CE/MS sprayer (p/n G1607B) is available.

The first part of this Technical Overview shows that the Agilent Jet Stream-compatible CE/MS sprayer can be used on a mass spectrometer equipped with a conventional ESI source without loss of sensitivity, compared to the standard sprayer. The second part compares the performance of the Agilent Jet Stream-compatible CE/MS sprayer on a MS system equipped with an Agilent Jet Stream source and a conventional ESI source, respectively. An equivalent or better sensitivity was obtained with the Agilent Jet Stream source.

Experimental

All CE separations were performed on an Agilent 7100 CE system. An Agilent 1260 Infinity Isocratic Pump equipped with degasser and 1:100 splitter was used for sheath liquid delivery. For CE and MS instrument control, Agilent ChemStation (revision B.04.03) and Agilent MassHunter (revision B.02.01, B2116.20, patches 1,2) installed on a single PC were employed. Prior to the actual measurements, optimal ion source parameters were determined by sample infusion. All measurement were repeated four times (n = 4).

Analysis of metamphetamine and methadone

Standard stock solutions in methanol of metamphetamine (MA), methadone (MTD) and their respective isotopically labeled derivatives at a concentration of 1,000 ppm were obtained from Lipomed (Arlesheim, Switzerland). Samples were prepared daily by diluting stock solutions in water.

CE conditions

Capillary:	Fused-silica capillary (BGB Analytik AG, Böckten, Switzerland), 50 µm id., total length 80 cm
Capillary coating:	CEofix dynamic coating compatible with MS detection (Analis, Namur, Belgium)
Background electrolyte (BGE):	25 mM ammonium acetate buffer, pH 5.0
Voltage:	+30 kV with a 0.3 minute voltage ramp
Temperature:	25 °C
Daily conditioning:	Flush (2 bars) with methanol for 5 minutes, water for 5 minutes
CEofix initiator and CEofix accelerator for 0.4 minutes each and BGE for 5 minutes	
Pre-run conditioning:	Flush (2 bars) with BGE for 3 minutes
Post-run conditioning:	Flush (2 bars) with CEofix accelerator 0.5 minutes; injection: sample, 50 mbar for 10 seconds and BGE, 50 mbar for 2 seconds

MS conditions

Instrument:	Agilent 6210 TOF; CE/MS
Sprayer version:	G1607A or G1607B
Ion source:	Dual ESI
Polarity:	ESI-positive
Fragmentor voltage:	150 V
Nebulizer gas pressure:	4 psi
Drying gas flow rate:	4 L/min
Drying gas temperature:	250 °C
Capillary voltage:	4,500 V
Sheath liquid:	Isopropanol-water-formic acid (50:50:0.5, v/v/v)
Sheath liquid flow rate:	3 µL/min
Scan rate:	2.5 spectra/s

Amino acid analysis

Samples were prepared daily by diluting an amino acid standard (1 nmol/µL, p/n 5061-3330) in water.

CE conditions

Capillary:	Bare-fused silica capillary, 50 µm id., total length 100 cm; BGE: 1 M formic acid;
Voltage:	+30 kV with a 0.3 minutes voltage ramp
Temperature:	20 °C
Pre-run conditioning:	Flush (1 bar) with BGE for 5 minutes
Injection:	Sample, 50 mbar for 8 seconds and BGE, 50 mbar for 2 seconds

MS conditions

Instrument:	Agilent 6320 TOF; CE/MS
Sprayer version:	G1607B
Ion source:	Agilent Jet Stream or Dual ESI
Polarity:	ESI-positive
Fragmentor voltage:	120 V
Nebulizer gas pressure:	10 psi
Drying gas temperature:	300 °C
Sheath liquid:	5 mM ammonium acetate in 50% methanol
Sheath liquid flow rate:	8 µL/min
Scan rate:	2 spectra/s

MS parameters for Agilent Jet Stream source only

Capillary voltage:	2,000 V
Nozzle voltage:	2,000 V
Drying gas flow rate:	8 L/min
Sheath gas temperature:	195 °C
Sheath gas flow rate:	3.5 L/min

MS parameters for Dual ESI source only

Capillary voltage:	4,000 V
Drying gas flow rate:	10 L/min

Organic acid analysis

Samples were prepared daily by diluting the organic acid test mixture (1,000 ppm, p/n 8500-6900) in water.

CE conditions

Capillary:	Bare-fused silica capillary, 50 µm id., total length 100 cm; BGE: 20 mM ammonium formate, pH 10
Voltage:	+30 kV with a 0.3-minutes voltage ramp
Temperature:	20 °C
Pre-run conditioning:	Flush (1 bar) with BGE for 5 min
Injection:	Sample, 50 mbar for 8 sec and BGE, 50 mbar for 2 seconds

MS conditions

Instrument:	6320 TOF, CE/MS
Sprayer version:	G1607B
Ion source:	Agilent Jet Stream or Dual ESI
Polarity:	ESI-negative
Fragmentor voltage:	100 V
Nebulizer gas pressure:	10 psi
Drying gas flow:	10 L/min
Drying gas temperature:	300 °C
Sheath liquid:	5 mM ammonium hydroxide in 50% methanol
Sheath liquid flow rate:	8 µL/min
Scan rate:	2 spectra/s

MS parameters for Agilent Jet Stream source only

Capillary voltage:	1,500 V
Nozzle voltage:	2,000 V
Sheath gas temperature:	195 °C
Sheath gas flow rate:	3.5 L/min

MS parameters for Dual ESI source only

Capillary voltage:	4,000 V

Results and discussion

Comparing the Agilent Jet Stream-compatible sprayer and the standard sprayer

The use of the Agilent Jet Stream source for CE/MS is only possible with a dedicated version of the CE/MS sprayer. This Agilent Jet Stream-compatible sprayer has a modified tip and needle design and can be visually distinguished from the standard sprayer in a straightforward manner (Figure 1). The Agilent Jet Stream-compatible sprayer can also be used in combination with a conventional ESI source. The performance of this sprayer in comparison to the standard CE/MS sprayer was tested with a toxicological sample on a TOF/MS system equipped with a Dual ESI source (Figure 2). Peak areas obtained for all compounds were up to two-fold higher with the Agilent Jet Stream-compatible sprayer over the whole concentration range tested. Signal-to-noise ratios confirmed this tendency (data not shown). Peak area repeatabilities were for all compounds 10% RSD or lower. Therefore, the Agilent Jet Stream-compatible sprayer in combination with a conventional ion source shows a slightly better performance than the standard sprayer. One reason for this better performance is the improved mechanical design of the Agilent Jet Stream-compatible sprayer that helps to position the sprayer needle exactly in the center of the sprayer body. This should, in turn, improve spray quality and therefore ionization and signal intensity.

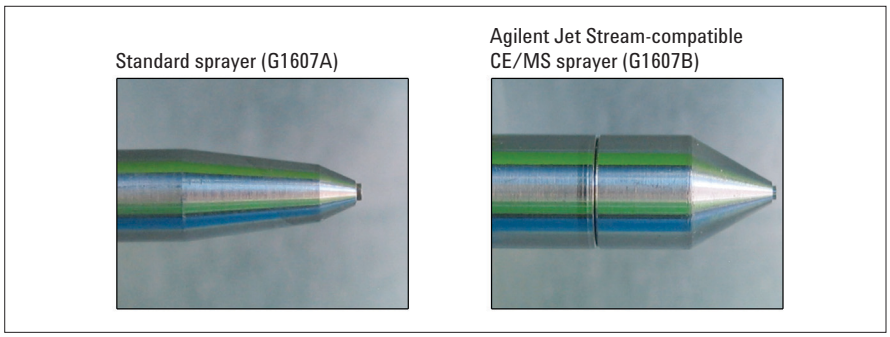

Figure 1
Photographs of the tips of different CE/MS sprayer versions.

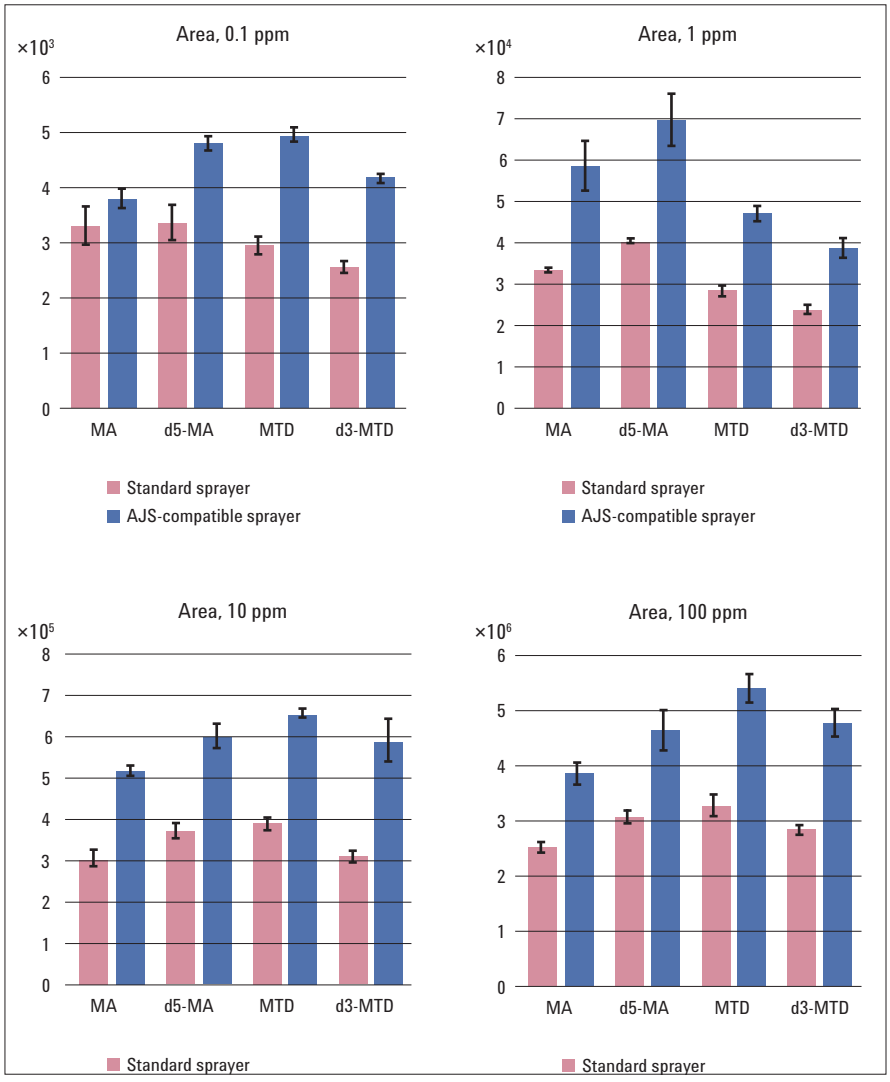

Figure 2
Comparison of standard and Agilent Jet Stream-compatible CE/MS sprayers on an MS instrument equipped with a Dual ESI source. Average peak areas (n = 4) with standard deviations obtained from extracted ion electropherograms of methadone, metamphetamine, and isotopically labeled derivatives at the indicated concentrations are shown. MA, Metamphetamine; d5-MA, d5-Metamphetamine; MTD, Methadone; d3-MTD, d3-Methadone.

Performance of the Agilent Jet Stream-compatible CE/MS sprayer in combination with different ion sources

A direct comparison of the performance of the Agilent Jet Stream-compatible sprayer on an MS instrument equipped with either an Agilent Jet Stream source or a conventional ESI source was done with small molecule standards in the positive and the negative ion mode. Figure 3 shows results obtained with an amino acid sample in the positive ion mode. On average, peak areas were slightly increased with the Agilent Jet Stream source. Peak area repeatabilities were 10% RSD or lower. With both ion sources, no background noise was observed for any amino acid except valine. To get an estimate of the sensitivity, different amino acid standard dilutions were analyzed down to a concentration of 0.01 µM. A similar number of detectable amino acids at given concentrations and therefore a similar sensitivity was observed with both ion source types (Figure 4). An at least equivalent sensitivity of the Agilent Jet Stream source in comparison to a conventional ESI source was obtained with sheath liquid flow rates in the range of 4–10 µL/min (data not shown).

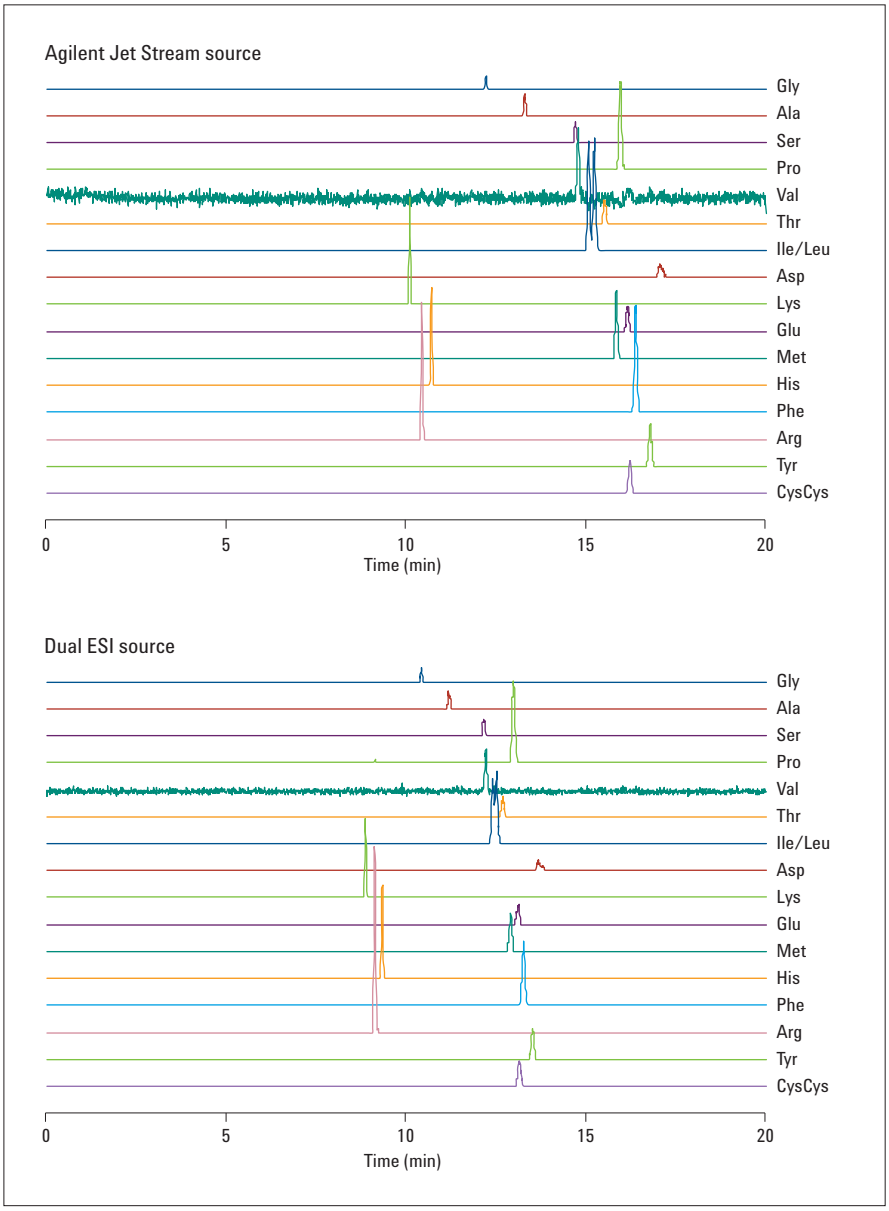

Figure 3
Performance of the Agilent Jet Stream-compatible CE/MS sprayer with different ion sources in the positive ion mode. Extracted ion electropherograms for all 17 amino acids present in the sample at a concentration of 10 µM are shown.

In summary, these data indicate a very comparable performance of the Agilent Jet Stream-compatible sprayer in combination with the Agilent Jet Stream source, versus the conventional Dual ESI sources in the positive ion mode. Results obtained with an organic acid sample in the negative ion mode are shown in Figure 5 and Table 1. In contrast to the results obtained in the positive ion mode, peak areas for all three sample compounds were 6–8 fold enhanced with the Agilent Jet Stream source. However, the signal-to-noise (s/n) was only similar or up to 4-fold increased due to the lower noise that was obtained with the Dual ESI source (Table 1). The performance of the Agilent Jet Stream compatible sprayer in combination with Agilent Jet Stream source in the negative ion mode was therefore improved, in comparison to the Dual ESI source for two out of three compounds tested.

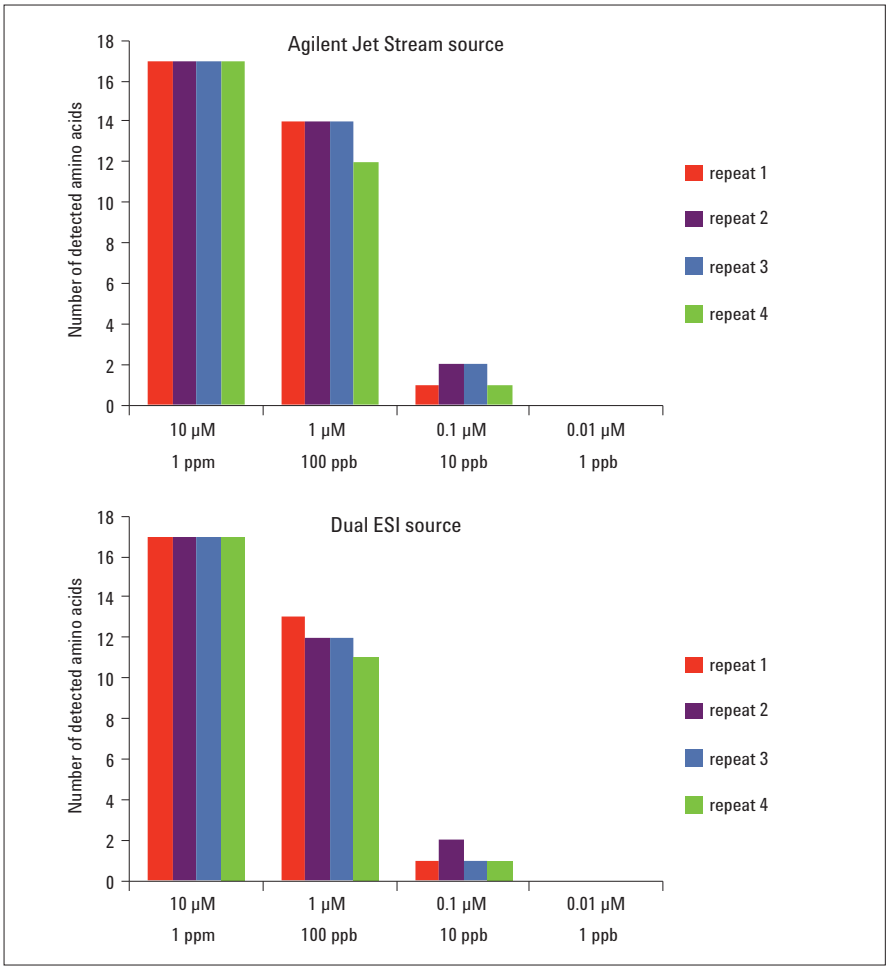

Figure 4
Limits of detection in the positive ion mode. Amino acid standard dilutions with concentrations of 10, 1, 0.1, and 0.01 µM were analyzed with different ion sources. The number of amino acids that could still be detected in the extracted ion electropherograms with a signal-to-noise ≥ 3 is shown. Concentrations in ppm/ppb units were calculated with a molecular weight of 100 g/mol.

Dual ESI source				
Analyte	**Area x 10^3**	**Area RSD%**	**s/n**	**s/n RSD%**
Lactic acid	7.0	3.1	3.5	9.4
Succinic acid	5.9	17.0	4.5	20.0
Malic acid	12.0	5.8	7.2	24.0
Agilent Jet Stream source				
Analyte	**Area x 10^3**	**Area RSD%**	**s/n**	**s/n RSD%**
Lactic acid	33	16.0	3.3	15.0
Succinic acid	49	8.8	8.4	17.0
Malic acid	77	6.3	26.0	12.0

Table 1
Results of the organic acid analysis in the negative ion mode. Shown are average peak areas and s/n with the corresponding repeatabilities (n=4) with different ion sources. Data were calculated from extracted ion electropherograms obtained at a sample concentration of 1 ppm. Peak-to-peak noise values were calculated over a 5 min interval in the vicinity of the peak of interest.

Sensitivity gains that can be obtained with the Agilent Jet Stream thermal gradient focusing technology in LC/MS are primarily due to more efficient ion drying and concentration by the superheated nitrogen sheath gas. However, these effects are flow rate dependent: a 5–10 fold increased sensitivity was seen with the Agilent Jet Stream source at flow rates ranging from 50 µL/min to 2.5 mL/min, with greatest gains typically at 0.25 to 1.0 mL/min[2]. The use of the Agilent Jet Stream source in combination with a dedicated microflow LC/MS sprayer enabled the extension of this range, sensitivity gains of 3–4 fold were reported for flow rates down to 15 µL/min[3,4]. However, typical flow rates for CE/MS employing the Agilent triple tube coaxial sheath flow interface are in the range of 1–10 µL/min. With the flow rate of 8 µL/min used, a substantial sensitivity gain was observed for only a fraction of the analytes tested in the negative ion mode, but not in the positive ion mode.

Furthermore, the best Agilent Jet Stream source parameters for CE/MS were found to be 195 °C and 3.5 L/min for sheath gas temperature and flow rate, respectively (cf. Experimental). These settings differ substantially from the standard values of 350 °C and 11 L/min that are recommended for best Agilent Jet Stream source performance in LC/MS[2]. These results may indicate that ion drying is not as critical for flow rates lower than 15 µL/min as compared to higher flow rates. Alternatively, principle differences in the design of LC/MS and CE/MS interfaces, for example, different sprayer tip dimensions, could limit the benefits that can be achieved with the Agilent Jet Stream thermal gradient focusing technology in CE/MS as compared to LC/MS.

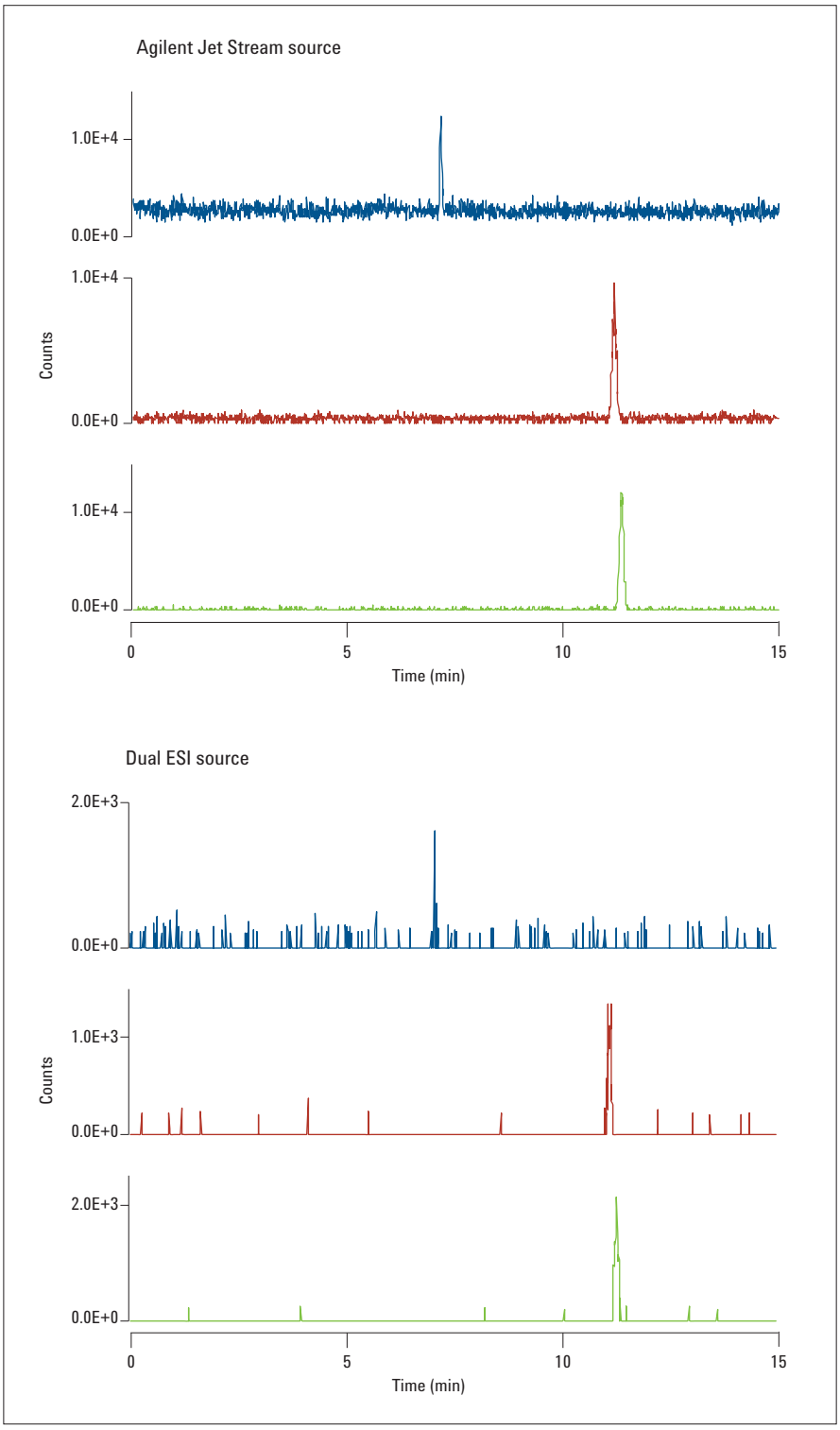

Figure 5
Performance of the Agilent Jet Stream-compatible CE/MS sprayer with different ion sources in the negative ion mode. Shown are extracted ion electropherograms for the three organic acids present in the sample at a concentration of 1 ppm (from top to bottom: lactic acid, succinic acid, and malic acid).

Conclusions

The Agilent Jet Stream thermal gradient focusing technology is compatible with CE/MS. The Agilent Jet Stream source can be used in combination with the G1607B version of the triple tube coaxial sheath-flow CE/MS sprayer. The Agilent Jet Stream-compatible sprayer fits as well into conventional ESI sources and showed a slightly improved performance as compared to the standard CE/MS sprayer. In a direct comparison of the performance of this sprayer on a MS instrument equipped with an Agilent Jet Stream source and a standard ESI source respectively, an at least equivalent or better sensitivity was obtained with the Agilent Jet Stream source.

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Appendix V.

Highly sensitive CE-ESI-MS/MS for accurate quantitation of drugs of abuse in bioanalysis using the Agilent 6490 Triple Quadrupole LC/MS System

I. Kohler, J. Schappler, S. Rudaz, M. Greiner, Agilent Technologies Application Note, publication number 5991-2395EN (2013)

Highly Sensitive CE-ESI-MS/MS for Accurate Quantitation of Drugs of Abuse in Bioanalysis Using the Agilent 6490 Triple Quadrupole LC/MS System

Application Note

Forensics and Toxicology

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Abstract

The combination of capillary electrophoresis and mass spectrometry (CE-MS) is particularly well suited to clinical and forensic toxicology due to its high separation efficiency, selectivity and sensitivity, short analytical time, and low solvent and sample consumption. A CE-ESI-MS/MS method was developed for the quantitation of drugs of abuse in urine samples with the highly sensitive Agilent 6490 Triple Quadrupole LC/MS system, including Jet Stream and ion funnel technologies, equipped with a triple-tube sprayer especially designed for CE hyphenation. Urines were simply diluted 10-fold prior to CE injection, and a pH-mediated stacking procedure was implemented to increase the loading capacity (20.5 % of the capillary length). This approach was found to increase the sensitivity of the method with limits of detection (LODs) as low as the ng/mL level. The quantitative procedure was validated for two model compounds, cocaine (COC) and methadone (MTD), according to SFSTP protocols and guidance of the Food and Drug Administration (FDA). Performance was evaluated for selectivity, response function, the limit of quantitation (LOQ), trueness, precision, and accuracy. COC was fully validated over a concentration range of 10–1,000 ng/mL, with accuracy included within the ± 30 % tolerance limits, as for MTD in the concentrations range of 21–1,000 ng/mL. The developed CE-ESI-MS/MS was eventually applied to real cases analysis.

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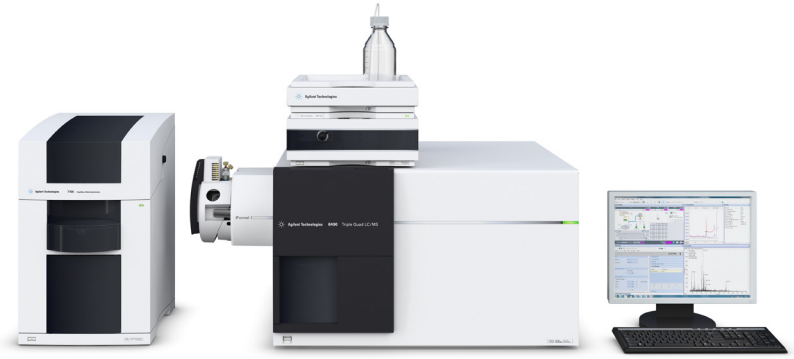
Introduction

The quantitation of xenobiotics in body fluids is of great importance in many fields, such as clinical and forensic toxicology, including therapeutic drug monitoring, metabolism studies, workplace drug testing, and doping analysis. CE represents an alternative technique to GC and LC for a large range of clinical and toxicological applications with numerous advantages, such as high separation efficiency, short analysis time, and low solvent and sample consumption. UV/Vis is the most widely used detection technique with CE configuration but suffers from a lack of sensitivity due to the narrow optical path length afforded by the internal diameter of the capillary. This lack of sensitivity, combined with the relatively low selectivity of UV/Vis detection, is considered a challenging issue for the determination of potentially low concentrated xenobiotics in body fluids. For quantitative purposes, CE can be hyphenated to various selective MS analyzers such as triple quadrupole in multiple reaction monitoring (MRM) mode. In this study, CE was hyphenated to a highly sensitive 6490 Triple Quadrupole LC/MS system equipped with Jet Stream and ion funnel technologies. Electrospray ionization (ESI) is the most widespread ionization source for coupling CE with MS and was used with the sheath-flow configuration. The sheath-flow interface is characterized by an additional make-up liquid flowing through a so-called triple-tube ESI sprayer that mixes with the CE effluent at the capillary tip, providing electrical contact at the outlet end, plus the appropriate flow rate (µL range) and solvent conditions for ionization of the analytes. A sprayer that presents an adapted design compared to the standard triple-tube sprayer, has been designed and was used for the hyphenation of CE with triple quadrupole.

Triple-Tube ESI Sprayer

The sheath-flow approach has been widely used due its stability, versatility, robustness, and ease-of-use. The commercial Agilent set-up positioned in an orthogonal configuration towards MS entrance derives from the LC/MS configuration. For CE-MS the LC-ESI sprayer has been replaced by a triple-tube sprayer containing two concentric tubes: one tube comprises the CE capillary outlet and transports the sheath liquid, surrounded by the other tube transporting the nebulizing gas. A second generation triple-tube sprayer G1607B has been commercialized with modifications of sprayer's tip and needle design that should help position the needle exactly in the center of the sprayer body. Figure 1 illustrates the earlier version CE-MS sprayer G1607A (Figure 1A) and the triple-tube sprayer G1607B (Figure 1B). The performance of both sprayers was evaluated on a set of toxicological compounds at various concentrations, and signal intensities were found to increase up to 1.5 fold with the new triple-tube sprayer, explained by an improvement of spray quality and, therefore, ionization and signal intensity[1].



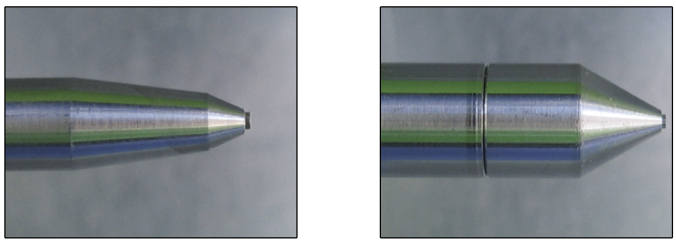


Figure 1. Illustration of CE-ESI-MS sprayer. A) Former triple-tube sprayer (p/n G1607A). B) New Triple-tube sprayer (p/n G1607B).

Agilent Jet Stream Technology

Modifications to the ESI source as well as the sprayer position were carried out to increase ionization and transmission efficiency, with adaptation in the position and number of heated gas inlets, the sprayer orientation relative to the sampling orifice, the diameter of MS orifice, and the number of transfer capillaries[2]. Some of these improvements were envisaged for CE-MS coupling. In this context, Agilent Jet Stream (AJS) thermal gradient focusing technology has been developed to improve (i) desolvation of nebulized spray droplets and (ii) better focus ions while keeping droplets away from the MS orifice. Both effects strengthen signal-to-noise (S/N) ratios and overall sensitivity. AJS consists of a modified ESI source with the addition of a collinear, concentric superheated nitrogen sheath gas surrounding the sprayer, confining the spray plume and increasing the desolvation efficiency. Due to an enhanced ion density in the confinement zone, more ions are available for sampling and, therefore, the overall ionization is more efficient. AJS was previously developed for LC/MS configuration but can be also used for CE-MS hyphenation, only with the G1607B triple-tube sprayer.

In a previous study, a direct comparison of the AJS source versus a conventional ESI source showed comparable performance on a set of low molecular weight compounds in positive ion mode[1].

Agilent iFunnel Technology

iFunnel technology encompasses (i) the AJS technology, (ii) a hexabore capillary, and (iii) a dual-stage ion funnel. On conventional Agilent mass spectrometers, ions produced in the source are initially transferred by a single inlet capillary of *ca.* 600 µm id restricting ion sampling. In a 6490 Triple Quadrupole LC/MS system, a short hexabore capillary assembly is used to increase the interface area of the MS inlet within the thermal ion confinement zone, leading to an increased ion sampling efficiency while maintaining good desolvation performance (Figure 2). Increasing the number of capillaries with the hexabore assembly simultaneously results in an increase of gas load in the mass spectrometer. A dual-stage ion funnel system composed of a series of closely-spaced ring electrodes, as illustrated in Figure 2, is added after the hexabore sampling capillary to remove the gas while increasing ion transmission, reducing neutral contaminants, and decreasing system noise[3,4]. Therefore, this iFunnel technology, which is available on some Agilent mass spectrometers, allows for an increased sensitivity compared to conventional ones.

This Application Note presents the development of a CE-ESI-MS/MS method for the quantitation of drugs of abuse in urine samples with CE hyphenated to the highly sensitive 6490 Triple Quadrupole LC/MS system. Urine samples were diluted 10-fold prior to CE injection and a pH-mediated stacking procedure was implemented to increase the loading capacity (20.5% of the capillary length) with an increase in sensitivity and LODs as low as the ng/mL level. Quantitative procedure was validated for two model compounds, cocaine (COC) and methadone (MTD), according to SFSTP protocols[5] and guidance of the FDA[6]. Performance was evaluated for selectivity, response function, the limit of quantitation (LOQ), trueness, precision, and accuracy. It was eventually applied to real cases.

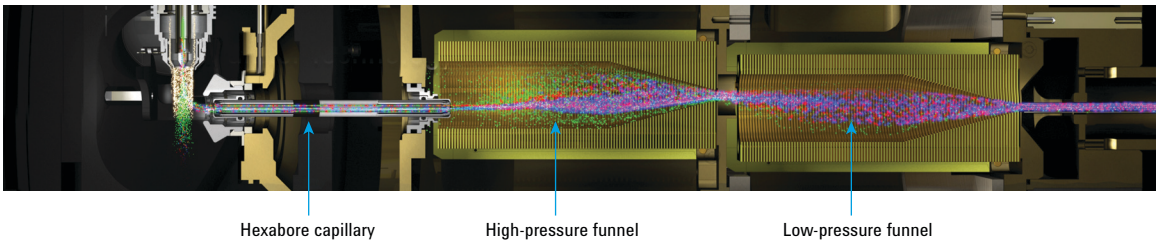


Figure 2. Hexabore capillary and dual-stage ion funnel[4].

Experimental

Chemicals

Hydrochloric acid, sodium hydroxide, analytical grade isopropanol (*i*-prOH), and 28 % ammonia solution (*m/v*) were obtained from Fluka (Buchs, Switzerland). Methanol (MeOH) and formic acid were purchased from Biosolve (Valkenswaard, Netherlands) and were all ULC/MS grade. Ultrapure water was supplied by a Milli-Q Advantage A10 purification system from Millipore (Bedford, MA, USA). Cocaine (COC), deuterated cocaine (d_3-COC), d,l-methadone (MTD) and deuterated d,l-methadone (d_3-MTD) in methanolic solutions (1 mg/mL) were obtained from Lipomed AG (Arlesheim, Switzerland).

Sample Preparation

Blank urine was obtained from a pool of six healthy Caucasian nondrug consumers and stored after collection in polypropylene tubes at –20 °C. Before analysis, the pooled urine was defrosted at ambient temperature, centrifuged at 10,000 rpm for 5 minutes and filtered through a 0.45-µm nylon filter (BGB Analytik AG, Böckten, Switzerland). Stock standard solutions of the solid analytes were prepared by dissolving each compound in MeOH to obtain a concentration of 1 mg/mL and stored at 4 °C until use. Blank pooled urine was spiked daily at desired concentrations. For this purpose, volumes of stock standard solutions were evaporated to dryness under a gentle steam of nitrogen and reconstituted in blank urine. Before injection, urine samples were diluted with BGE and water (1:1:8, *v/v/v*).

Calibration standards (CS) were independently prepared in blank pooled urine on each of the three validation series (j = 3) at three known concentrations (10, 500, and 1,000 ng/mL for both compounds, k = 3) with two replicates for each concentration (n = 2). d_3-COC and d_3-MTD were spiked in each sample at a concentration of 50 ng/mL, and calibration curves were built from the peak areas of COC and MTD *versus* the peak areas of d_3-COC and d_3-MTD, respectively.

Validation standards (VS) were independently prepared in blank pooled urine for each of the three validation series (j = 3) at four known concentrations (10, 25, 500, and 1,000 ng/mL for both compounds, k = 4), with four replicates for each concentration (n = 4). d_3-COC and d_3-MTD were spiked in each sample at a concentration of 50 ng/mL, and reported signals were obtained from the peak areas of COC and MTD *versus* the peak areas of d_3-COC and d_3-MTD, respectively.

Toxicological samples were received from the Laboratory of Clinical Chemistry (Geneva Hospitals, Geneva, Switzerland) and stored at – 20°C until use. Before analysis, samples were treated in the same manner in which the blank pooled urine was treated, and IS were spiked at 50 ng/mL before dilution and injection. Two independent analyses were performed for each sample (N = 2).

Instrumentation

CE experiments were performed with an Agilent 7100 CE system, equipped with an integrated diode array detector, an autosampler and a power supply able to deliver up to 30 kV. Separation was performed using a fused-silica capillary (BGB Analytik AG, Böckten, Switzerland) with a total length of 80 cm and a 50 µm id. Before its first use, the capillary was rinsed sequentially at 2 bar with MeOH (5 minutes), water (3 minutes), 0.1 M HCl (5 minutes), water (3 minutes), 1 M NaOH (5 minutes), and water (3 minutes). The capillary was conditioned daily with MeOH (5 minutes), water (5 minutes), and BGE (10 minutes) at 2 bar. Prior to each sample injection, the capillary was rinsed at 2 bar with BGE (3 minutes). When not in use, the capillary was rinsed with water and dry-stored. Samples were kept at ambient temperature in the autosampler. The CE instrument was coupled to a 6490 Triple Quadrupole LC/MS system through a coaxial sheath flow AJS interface. CE-ESI-MS conditions are presented in Table 1.

Table 1. CE-ESI-MS conditions.

Capillary electrophoresis	
Injection preplug	7 % NH_4OH (*m/v*) at 50 mbar for 10 s (0.7 % of capillary length)
Sample injection	at 100 mbar for 150 seconds (20.5 % of capillary length)
Injection postplug	BGE at 50 mbar for 3 seconds (0.2 % of capillary length)
BGE	1 M formic acid, pH 1.8
Separation voltage	30 kV with initial ramping of 833 V/s (36 seconds)
Temperature	25 °C
Mass spectrometry	
Sheath liquid	*i*-PrOH-water-formic acid (50:50:0.5, *v/v/v*) at 5 µL/min
Drying gas temperature	200 °C
Drying gas flow rate	16 L/min
Nebulizing gas pressure	8 psi
Sheath gas temperature	200 °C
Sheath gas flow rate	3.5 L/min
Nozzle voltage	2,000 V
Capillary voltage	2,000 V
EMV	300 V
Fragmentor voltage	380 V
Dwell time	80 ms
Mass resolution	0.7 u

Table 2 shows MRM transitions for COC, d3-COC, MTD, and d3-MTD with their respective collision energy and cell accelerator voltage.

Results and Discussion

Method Development

In toxicology, a fast, sensitive, and accurate quantitation method is required. In this case, tedious and time-consuming sample preparations, for example, liquid-liquid extraction (LLE), or solid-phase extraction (SPE) procedures are preferably skipped while a simple dilution is promoted if possible. A stacking procedure was implemented to increase the volume injected and to offset the loss of sensitivity caused by urine dilution. In contrast to other stacking procedures, which can be strongly dependent on the saline composition of the sample, a pH-mediated stacking procedure was applied. Samples were diluted 10-fold with BGE and water (1:1:8, *v/v/v)* prior to injection. This dilution allowed for (i) the normalization of urine pH, (ii) a full ionization of analytes before injection, and (iii) a consequent decrease of the sample conductivity. A small preplug of 7 % NH_4OH (*m/v*), corresponding to 0.7 % of the capillary length, was injected prior to acidified diluted urine sample, providing stacking without disrupting the electrophoretic process. When applying the separation voltage, analytes under cationic form migrated until they reached the strong alkaline zone, became neutral, and stacked in a narrow zone at the boundary of the sample and the alkaline plug. The latter was then acidified by the BGE and the analytes returned to a cationic state and began their electrophoretic migration[7]. With the developed pH-mediated stacking procedure, 20.5 % of the capillary length was filled during injection without any peak broadening. Figure 3 illustrates the improvement for COC in loading capacity obtained when injecting 20.5 % of the capillary length with pH-mediated stacking (Figure 3B) compared to the conventional hydrodynamic injection of 1.0 % of the capillary length without sample stacking (Figure 3A). With these conditions, LODs (expressed as the concentration where the S/N ratio was superior to 3) were estimated at 2 ng/mL for MTD and COC.

Table 2. MRM transitions for COC, d_3-COC, MTD, and d_3-MTD.

Compound	Precursor ion	Product ion	Collision energy	Cell accelerator voltage
COC	304.1 *m/z*	182.0 *m/z*	20 eV	3 V
d_3-COC	307.1 *m/z*	185.0 *m/z*	20 eV	3 V
MTD	310.2 *m/z*	265.1 *m/z*	10 eV	3 V
d_3-MTD	313.2 *m/z*	268.1 *m/z*	10 eV	3 V

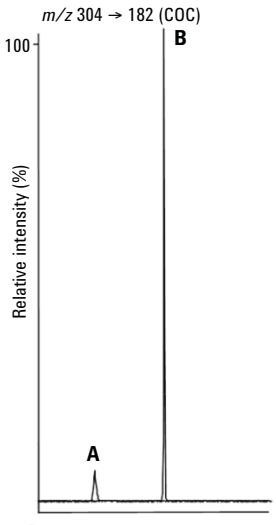


Figure 3. Example of pH-mediated stacking for COC at 100 ng/mL. A) 1% HD injection, without stacking. B) 20.5% HD injection with pH-mediated stacking.

Selectivity

With urine dilution, endogenous compounds that may alter the analyte ionization process (signal suppression or enhancement) are still present. The matrix effect (ME) was evaluated for COC and MTD to determine the influence of potential comigrating interferents on analyte ionization prior to triple quadrupole determination. For this purpose, a procedure based on the methodology proposed by Matuszewski *et al*. for the quantitation of ME in biological fluids was implemented[8]. COC did not show any significant suppression, with an ME of 92 ± 9% (± 2SD), while a relevant signal suppression was observed for MTD, with an ME of 73 ± 5%. Therefore, the use of deuterated IS correction for an external calibration within the reconstituted matrix was selected for quantitation.

The method selectivity was also evaluated by comparing electropherograms obtained by injecting (i) blank urine (CAL 00), (ii) urine spiked with d_3-COC and d_3-MTD at 50 ng/mL (CAL 0), and (iii) a VS at 25 ng/mL for COC and MTD, with the IS set at 50 ng/mL. As illustrated in Figure 4, for both analytes, no interference was detected at the migration times corresponding to COC and MTD or their respective IS.

Validation

SFSTP validation guidelines[5] were followed to evaluate the quantitative performance of the developed CE-ESI-MS/MS method for COC and MTD analysis on three independent series (j = 3). The validation protocol involved three concentrations (k = 3) with two repetitions (n = 2) for CS and four concentrations (k = 4) with four repetitions (n = 4) for VS. The concentrations' ranges for COC and MTD were determined according to the standard concentrations detected in samples from drug consumers. A concentration range of 10–1,000 ng/mL was selected for both compounds. CS and VS were prepared in blank pooled urine. Several regression models for calibration curve adjustment were evaluated. Trueness (relative bias) and precision were assessed for each concentration level. Precision was estimated with the variances of repeatability (s^2r) and intermediate precision (s^2R), and was expressed by RSD (%). Confidence intervals were calculated with fixed degrees of freedom ($df = k \cdot j - n$) at a risk α = 5 %. Accuracy profiles for COC and MTD were built with trueness and upper and lower confidence limits, with the latter two representing the total error of the method.

Response function

Different regression models were assessed for the calibration curve, including:

- Ordinary least square (OLS) regression
- OLS after square root transformation of concentrations (x) and responses (y)
- OLS after logarithm transformation of concentrations (x) and responses (y)
- OLS forced through the origin, external standard with high level of CS, and weighting least square with two weighting factors (1/x and $1/x^2$).

For all of the calibration models, accuracy profiles were plotted for COC and MTD.

The optimal regression model was selected according to the best total error profile obtained when covering the whole concentration range. OLS after square root transformation was selected as the best calibration model for COC with confidence intervals contained inside the acceptance limits, which were set at ± 30% according to the guidelines for quantitation in bioanalysis[9]. For MTD, the best model for calibration was the OLS after square root transformation, but the lowest VS (10 ng/mL) was outside of the acceptance limits. The LOQ was, therefore, established on the basis of the accuracy profile, as discussed below.

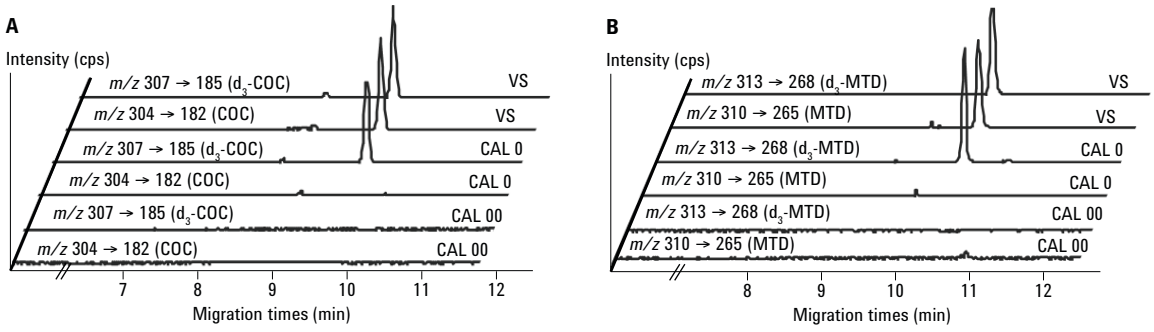


Figure 4. Evaluation of the method selectivity. Electropherograms obtained A) for COC, B) for MTD.

Trueness and precision

The trueness of an analytical procedure expresses the closeness of agreement between the mean values obtained from a series of measurements and the true values[5]. The results for trueness were assessed from the VS by relative bias (%) and are presented in Table 3. For COC, the relative biases were all satisfactory, as they did not exceed the threshold of ± 15 %. For MTD, the relative biases for medium and high concentrations, that is, 25, 500, and 1,000 ng/mL, were lower than ± 2 %. However, with a relative bias of 38.5 %, the lowest concentration (10 ng/mL) was unacceptable.

The precision of the method was estimated using the repeatability and intermediate precision at each VS and was expressed by RSDs. Table 3 shows that the RSD values for COC were in the range of 3.0–5.7 % for both repeatability and intermediate precision, showing strong precision in the developed method. For MTD, unsatisfactory RSD values of 21.0 and 27.8 % were obtained at 10 ng/mL for repeatability and intermediate precision, respectively, while the RSDs were lower than 7.1 % for higher concentrations.

Table 3. Validation criteria and results for COC and MTD in urine (j = 3, k = 4, n = 4).

Validation criterion	COC	MTD
Trueness		
Relative bias (%)		
10 ng/mL	12.0	38.5
25 ng/mL	1.0	1.7
500 ng/mL	-3.1	-1.8
1,000 ng/mL	0.1	0.7
Precision		
Repeatability/intermediate precision [RSD, in %]		
10 ng/mL	5.7/5.7	21.0/27.8
25 ng/mL	5.0/5.0	7.1/7.1
500 ng/mL	4.1/4.1	2.8/3.6
1,000 ng/mL	3.0/3.3	3.0/3.0
Accuracy		
Lower/upper confidence limits of the total errors [%]		
10 ng/mL	-1.1/25.1	-25.5/102.5
25 ng/mL	-10.5/12.4	-14.7/18.0
500 ng/mL	-12.5/6.3	-10.1/6.5
1,000 ng/mL	-7.4/7.6	-6.2/7.6
lower limit of quantitation LLOQ (ng/mL)	10	21

Accuracy

Accuracy is the expression of the total error of the analytical method and was chosen to evaluate the capacity of the developed analytical method to quantify samples with an accepted risk of α = 5 %[6,10]. The relative accuracy profiles for COC and MTD are shown in Figure 5. The lower and upper confidence limits of the mean bias (%) for COC, as shown in Table 3, were included within the acceptance limits of ± 30 % for each level of concentration. The developed method is, therefore, accurate for the quantitation of COC over the investigated concentration range (10–1,000 ng/mL). The lowest concentration level (10 ng/mL) was confirmed to be the LLOQ, which is defined by the smallest quantity of analyte that can be quantified with a defined accuracy within the acceptance limits.

For MTD, the LLOQ was interpolated from the absolute accuracy profile of MTD and defined at 21 ng/mL because the lowest concentration level (10 ng/mL) was not included within the acceptance limits of ± 30 %. With this LLOQ, the quantitation of MTD was found to be accurate in the range of 21–1,000 ng/mL.

Application to Real Cases

COC and MTD contained in two toxicological samples coming from the Laboratory of Clinical Chemistry (Geneva Hospitals, Switzerland) were quantified with the developed method. A calibration curve was constructed the same day (k = 3, n = 2) and OLS was applied after square root transformation of concentrations and responses. The confidence interval associated to the mean results is expressed with Equation 1.

Equation 1.

$$\bar{x} \pm t_{df,\alpha} \sqrt{\frac{s^2r}{N} + s^2g}$$

$\bar{x}$ is the mean result and N is the number of analyses. $t_{df,\alpha}$ (Student constant dependent on α and df), s^2r and s^2g were determined during a validation with regular ANOVA-based variance decomposition. Because most of the variability came from repeatability (s^2r) and not from the interseries variance (s^2g), two replications (N = 2) were performed to reduce the intraday variability and to obtain a narrow confidence interval for the final result. In the first sample, COC concentration was found to be 41.0 ± 6.4 ng/mL.

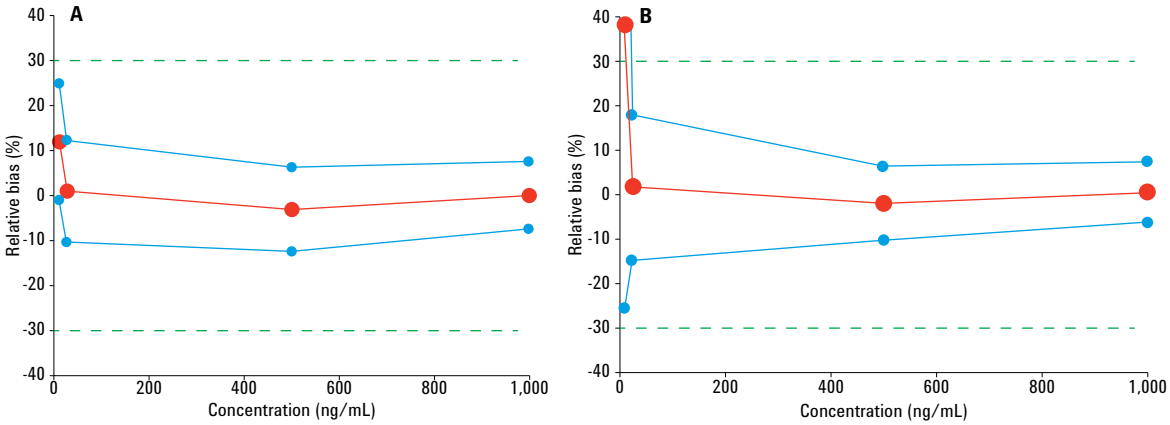


Figure 5. Accuracy profiles obtained for A) COC, and B) MTD in urine. Green dashed lines express ± 30 % acceptance limits

Figure 6A presents the corresponding electropherograms. This relatively low concentration can be related to a low dose (for example, less than ca. 10 mg of crack, intranasal, or intravenous dose) and/or a late urine collection (> 24 hours) after COC consumption. In the second sample, 462.9 ± 33.5 ng/mL of MTD were detected. Figure 6B shows the electropherograms obtained for the second sample. Due to the relatively long detection time window of MTD in urine and the high CYP450 inter-individual, this concentration can be related to both initial and maintenance MTD treatment.

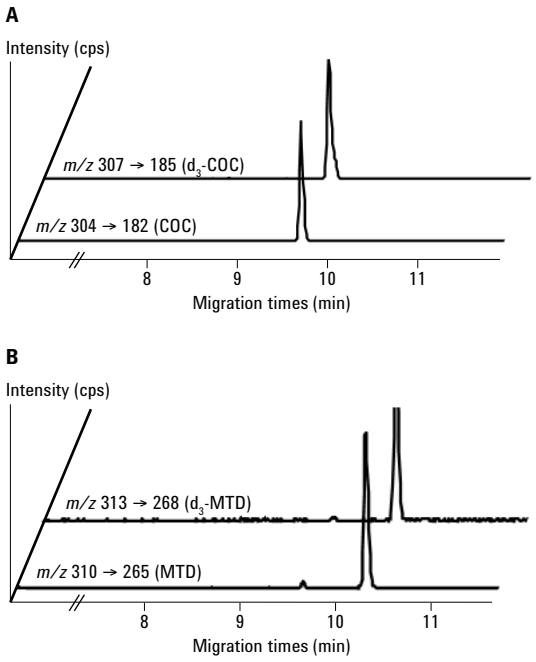


Figure 6. Electropherograms obtained for toxicological samples. A) Sample no. 1 containing COC, B) Sample no. 2 containing MTD.

Conclusion

A fast, selective and sensitive CE-ESI-MS/MS method was developed for the quantitation of drugs of abuse in urine with the highly sensitive Agilent 6490 Triple Quadrupole LC/MS system equipped with a triple-tube sprayer, as well as Jet Stream and ion funnel technologies. Urine samples were diluted 10-fold prior to CE injection, avoiding a tedious and time-consuming sample preparation, and a pH-mediated stacking procedure was implemented to increase the loading capacity (20.5% of the capillary length). The combination of this online preconcentration with the highly sensitive 6490 Triple Quadrupole LC/MS system led to LODs as low as the ng/mL level in urine.

A validation procedure based on accuracy profiles was applied to assess the quantitative performance of the developed CE-ESI-MS/MS. Selectivity, response function, the LLOQ, trueness, precision, and accuracy were estimated for two model and common drug of abuse, COC and MTD. COC was fully validated over the range of concentrations of 10–1,000 ng/mL with accuracy included within the ± 30% tolerance limits, as for MTD in the concentrations range of 21–1,000 ng/mL.

The developed CE-ESI-MS/MS method was successfully applied to real toxicological samples.

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