Evolution of sub/supercritical fluid chromatography – mass spectrometry for the analysis of highly polar compounds and biological matrices

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Les Facultés de médecine et des sciences, sur le préavis de Monsieur J.-L. VEUTHEY, professeur ordinaire et directeur de thèse (Section des sciences pharmaceutiques), Monsieur D. GUILLARME, docteur et codirecteur de thèse (Section des sciences pharmaceutiques), Monsieur J.-L. WOLFENDER, professeur ordinaire (Section des sciences pharmaceutiques), Madame C. WEST, professeure associée (Département de biologie cellulaire), Monsieur F. BOTRE, professeur associé (Institut des sciences du sport, Université de Lausanne, Lausanne), Monsieur E. REGALADO, docteur (Analytical Research and Develpment, Merck & Co, Inc.- Rahway, United Sates of America), Monsieur J.-C. SANCHEZ, professeur associé (Faculté de médecine), autorisent l'impression de la présente thèse, sans exprimer d'opinion sur les propositions qui y sont énoncées.

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Résumé de la thèse

Ce travail de thèse a été réalisé dans le groupe d'Analyse Pharmaceutique de l'Unité des Sciences Analytiques, entité appartenant à l'Institut des Sciences Pharmaceutiques de Suisse Occidentale, Université de Genève. Ce travail a été réalisé sous la supervision du Professeur Jean-Luc Veuthey et la co-supervision du Docteur Davy Guillarme. Les principaux domaines d'expertise couverts par l'Unité des Sciences Analytiques s'articulent autour de l'étude et de la mise au point des aspects fondamentaux des techniques de séparation (tant chromatographiques, qu'électrophorétiques), ainsi que du développement de procédures de préparation d'échantillons pour l'analyse de différentes matrices biologiques (par exemple, urine, plasma, cultures cellulaires, etc.) et, enfin, de la mise en œuvre de différents détecteurs, principalement spectrophotomètre ultra-violet (UV) et spectromètre de masse (MS). L'ensemble de ces outils est utilisé pour caractériser une vaste catégorie de molécules, allant de petits médicaments ou métabolites pharmaceutiques à des formulations biopharmaceutiques plus complexes incluant des anticorps monoclonaux ou des oligonucléotides. Par conséquent, l'unité des sciences analytiques se trouve impliquée dans de nombreux domaines d'application, tels que le contrôle antidopage, la toxicologie, le métabolisme des médicaments, la métabolomique et l'analyse des produits pharmaceutiques et biopharmaceutiques.

Au sein de l'Unité des Sciences Analytiques, le groupe d'Analyse Pharmaceutique se concentre sur le développement et la compréhension des aspects fondamentaux de la plupart des techniques chromatographiques, en se focalisant sur la chromatographie liquide (LC) et, plus particulièrement, sur ses différents modes tels que la chromatographie liquide à polarité des phases inversées (RPLC), la chromatographie liquide d'interaction hydrophile (HILIC), la chromatographie d'exclusion de taille (SEC), la chromatographie d'échange d'ions (IEX), la chromatographie d'interaction hydrophobe (HIC). L'application de ces approches chromatographiques pour l'analyse des petites et grosses molécules a fait l'objet de nombreuses thèses menées dans ce groupe de recherche, comme en témoigne la thèse du Docteur Alexandre Goyon intitulée " Evaluation de méthodes chromatographiques et électrophorétiques pour la caractérisation de protéines thérapeutiques " (Université de Genève, Thèse n°10, 2019), du Docteur Aurèlie Périat intitulée "Utilisation de la chromatographie HILIC pour l'analyse de composés d'intérêt pharmaceutique" (Université de Genève, Thèse n°4871, 2015), ou du Docteur Dao Nguyen intitulée "Analyses rapides et ultra-rapides en chromatographie liquide : application aux composés pharmaceutiques" (Université de Genève, Thèse n°3862, 2007). Cependant, la chromatographie liquide n'est pas la seule

option analytique disponible dans les laboratoires. Des techniques orthogonales, comme l'électrophorèse capillaire (CE), ont fait l'objet d'autres thèses menées dans ce groupe, comme le travail du Docteur Isabelle Kohler intitulé " Sensitivity Improvements in Capillary Electrophoresis - Mass Spectrometry for Clinical and Forensic Toxicology " (Université de Genève, Thèse n°4611, 2013), ou celui réalisé par le Docteur Aline Staub intitulé "Analyse de protéines intactes par électrophorèse capillaire couplée à un spectromètre de masse à temps de vol" (Université de Genève, Thèse n°4288, 2011).

Un troisième type de technique séparative est également à la disposition des analyticiens. La chromatographie en fluide supercritique (SFC) est une technique de séparation présentant une complémentarité et une orthogonalité à la LC. Développée initialement dans les années 1960, la SFC a démontré au cours des années qu'elle devait être considérée comme une alternative valable aux séparations LC, en particulier dans les exemples où la LC ne fournit pas une séparation satisfaisante. Dans le groupe d'Analyse Pharmaceutique, la SFC a déjà fait l'objet de différentes recherches et thèses, comme le travail du Docteur Alexandre Grand-Guillaume Perrenoud intitulé "Chromatographie en phase supercritique : Nouvelles perspectives dans le domaine de l'analyse pharmaceutique " (Université de Genève, Thèse n°4717, 2014), et celui du Docteur Vincent Desfontaine nommé " L'utilisation de la chromatographie en phase supercritique moderne et de la chromatographie en phase supercritique - spectrométrie de masse dans l'analyse pharmaceutique " (Université de Genève, Thèse n°5181, 2018). Dans ces deux travaux de thèse, les performances de la SFC ont été discutées, en utilisant la dernière génération de systèmes SFC sortie au début des années 2010, également connue sous le nom de chromatographie en phase supercritique à ultra-haute performance (UHPSFC). Il a été démontré que cette nouvelle instrumentation de pointe, ainsi que la commercialisation de phases stationnaires UHPSFC dédiées, fonctionnalisées sur des particules de silice de moins de 2 µm, ont permis à cette technique de devenir une alternative fiable à la LC. De plus, le couplage de l'UHPSFC à la MS a été étudié de manière approfondie, en évaluant la compatibilité ainsi que les différentes stratégies de couplage. Enfin, quelques applications de l'UHPSFC-UV et de l'UHPSFC-MS ont été étudiées et développées avec succès, principalement pour l'analyse des impuretés pharmaceutiques ainsi que dans le contexte du contrôle anti-dopage.

Le but de cette thèse est d'étendre le travail déjà effectué par les deux précédents projets de recherche, en se concentrant sur l'étude des performances et de la robustesse de l'UHPSFC-MS dans les laboratoires de routine. En outre, l'analyse de composés hautement polaires grâce à l'UHPSFC a été testée, afin de vérifier l'attrait potentiel de cette technique non seulement comme alternative à la RPLC mais aussi au mode HILIC. À cette fin, l'UHPSFC a été appliquée dans différents domaines, tels que la mètabolomique non ciblée utilisant

différentes matrices biologiques, et l'analyse de peptides. Enfin, certains aspects fondamentaux de l'UHPSFC tels qu'ont également fait l'objet de travaux, dans le but de compléter les connaissances sur les mécanismes contrôlant cette technique.

Abstract

This thesis has been conducted in the group of Pharmaceutical Analysis of the Analytical Sciences Unit, an entity that belongs to the Institute of Pharmaceutical Sciences of Western Switzerland, University of Geneva. This work was accomplished under the supervision of Professor Jean-Luc Veuthey and the co-supervision of Doctor Davy Guillarme. The main areas of expertise covered by the Analytical Sciences Unit revolve around the investigation and focus on the fundamental aspects of separation techniques (both chromatographic and electrophoretic), as well as on the development of sample preparation procedures for different biological matrices (e.g., urine, plasma, cell cultures, etc.) and, finally, on the implementation of different detectors, mainly ultra-violet (UV) and mass spectrometers (MS). The ensemble of these tools is used to perform a variety of analyses in several domains, ranging from small pharmaceutical drugs or metabolites to large biopharmaceutical formulations including monoclonal antibodies and oligonucleotides. Consequently, the Analytical Sciences Unit finds itself involved in many application areas, such as anti-doping control, toxicology, drug metabolism, metabolomics and the analysis of pharmaceuticals and biopharmaceuticals.

Within the Analytical Sciences Unit, the group of Pharmaceutical Analysis focuses in the development and understanding of the fundamental aspects for most of the chromatographic techniques, concentrating on liquid chromatography (LC) and, specifically, on its different modes such as reversed phase liquid chromatography (RPLC), hydrophilic interaction liquid chromatography (HILIC), size exclusion chromatography (SEC) and ion-exchange chromatography (IEX). Its application to both small and large molecules has been the subject of many thesis conducted in this research group, as demonstrated by the thesis of Doctor Alexandre Goyon entitled "*Evaluation de méthodes chromatographiques et électrophorétiques pour la caractérisation de protéines thérapeutiques*" (University of Geneva, Thesis n°10, 2019), of Doctor Aurèlie Périat entitled "*Utilisation de la chromatographie HILIC pour l'analyse de composés d'intérêt pharmaceutique*" (University of Geneva, Thesis n°4871, 2015), finally of Doctor Dao Nguyen entitled "*Analyses rapides et ultra-rapides en chromatographie liquide : application aux composés pharmaceutiques*" (University of Geneva, Thesis n°3862, 2007). However, LC is not the only analytical option available in laboratories. Orthogonal techniques, such as capillary electrophoresis (CE), have been the subject of other theses conducted in this group, such as the work of Doctor Isabelle Kohler entitled "*Sensitivity Improvements in Capillary Electrophoresis – Mass Spectrometry for Clinical and Forensic Toxicology*" (University of Geneva, Thesis n°4611, 2013), or the one performed by Doctor Aline Staub

entitled "*Analyse de protéines intactes par électrophorèse capillaire couplée à un spectromètre de masse à temps de vol*" (University of Geneva, Thesis n°4288, 2011).

A third analytical approach is also available to researchers. Supercritical Fluid Chromatography (SFC) is a separation technique presenting a complementarity and orthogonality to LC. Initially developed in the 1960s, SFC has demonstrated throughout the course of many years that it should be considered a valid alternative to LC separations, especially in examples where LC does not provide a satisfactory separation. In the Pharmaceutical Analysis group, SFC has been already the subject of different investigations and of different theses, such as the work of Doctor Alexandre Grand-Guillaume Perrenoud entitled "*Chromatographie en phase supercritique : Nouvelles perspectives dans le domaine de l'analyse pharmaceutique*" (University of Geneva, Thesis n°4717, 2014), and the following one of Doctor Vincent Desfontaine named "*The use of modern supercritical fluid chromatography and supercritical fluid chromatography – mass spectrometry in pharmaceutical analysis*" (University of Geneva, Thesis n°5181, 2018). In these two works, the performance of SFC was discussed, using the latest generation of chromatographic systems released in the early 2010s, also known as ultra-high performance supercritical fluid chromatography (UHPSFC). It was discovered that the new state-of-the-art instrumentation, together with the commercialization of dedicated UHPSFC stationary phases functionalized on sub-2 µm silica particles, has enabled this technique to become a reliable and valid alternative to LC. Moreover, the hyphenation of UHPSFC to MS was thoroughly investigated, assessing their compatibility as well as different strategies in their coupling. Finally, some applications of UHPSFC-UV and UHPSFC-MS have been investigated and successfully developed, mainly on the analysis of pharmaceutical impurities as well as in the context of anti-doping control.

The scope of this thesis has been to expand the work already performed by the previous two research projects, focusing on the investigation of the performance and robustness of UHPSFC-MS in routine laboratories. Furthermore, the analysis of highly polar compounds was tested using UHPSFC, to verify the potential attractiveness of this technique not only as an alternative to RPLC but also to HILIC analysis, in which strongly polar and hydrophilic substances are analyzed. To this purpose, UHPSFC has been applied in different fields, such as untargeted metabolomics employing different biological matrices and peptide analysis. Finally, some fundamental aspects of UHPSFC have been also the subject of work, in an attempt to complement the knowledge on the mechanisms controlling this technique.

Structure of the thesis

This PhD work is structured in three sections.

In Chapter I, an exhaustive description of the latest trends in UHPSFC in the literature is given. A particular focus is put on the choice of the organic modifier and additives in the mobile phase as well as in the different gradient profiles used nowadays in UHPSFC, with a specific focus on the analysis of highly polar analytes. In addition, details on how UHPSFC has been assessed in terms of method validation and method robustness are given. In the second part, an overview of the hyphenation between UHPSFC and MS is described, together with the latest applications involving the combined use of these two techniques.

Chapter II is divided into four sections. In the first one, an evaluation of the reproducibility of an UHPSFC method, hyphenated to tandem MS, is discussed. To do so, a generic UHPSFC-MS/MS method was developed for the analysis of a library of anti-doping agents, as standards and spiked in human urine. The performance of this approach, in terms of robustness and variability of the retention times of the considered analytes, was investigated. In the initial work, all experiments were performed in a single laboratory. In the subsequent project, the interlaboratory variability of this technique was taken into consideration. In the second section, some theoretical considerations are given on the use of unconventional temperatures (from -5°C to 80°C) in UHPSFC, employing a new gradient profile called Unified Chromatography (UC). A focus was made on how the performance of UHPSFC are impacted by the change in the column temperature at four different percentages of the organic modifier. In the third section, the applicability of UHPSFC in the field of metabolomics was evaluated. A first look is given at the optimization of the chromatographic conditions needed to ensure the simultaneous analysis of apolar and polar compounds, using a restricted library of 57 metabolites as standards. Subsequently, a scale up to 600 metabolites was performed in the second part of this metabolomics work. An evaluation of how biological matrices, such as human urine and plasma, impact the quality of the UHPSFC-HRMS method is also provided. In the fourth section, the performance of UHPSFC for peptide analysis was evaluated. A comparison to UHPLC is given, using a set of 12 synthetic and 6 commercial peptides. The retention patterns of the two separation techniques was discussed and compared. Finally, the scale-up of the UHPSFC method to a semi-preparative scale was evaluated, thanks to a collaboration established with the Analytical Research and Development department at Merck & Co, Inc. (Rahway, NJ, USA).

Articles & communications

The present thesis has been subjected to publications in peer-reviewed scientific journals. Research projects have been also presented at national and international congresses, in the form of either oral or poster communications.

Peer-reviewed scientific articles

I. *Applicability of supercritical fluid chromatography – mass spectrometry to metabolomics. I – Optimization of separation conditions for the simultaneous analysis of hydrophilic and lipophilic substances.*
V. Desfontaine, G.L. Losacco, Y. Gagnebin, J. Pezzatti, W.P. Farrell, V. González-Ruiz, S. Rudaz, J.-L. Veuthey, D. Guillarme; J. Chromatogr. A, 1562 (2018) 622 96-107.

II. *Supercritical fluid chromatography - mass spectrometry: recent evolution and current trends*.
G.L. Losacco, J.-L. Veuthey, D. Guillarme; Trends Anal. Chem. 118 (2019), 731-738

III. *Supercritical fluid chromatography – mass spectrometry in routine anti-doping analyses: estimation of retention time variability under reproducible conditions.*
G.L. Losacco, E. Marconetto, R. Nicoli, T. Kuuranne, J. Boccard, S. Rudaz, J.-L. Veuthey, D. Guillarme, J. Chromatogr. A, 1616 (2020), 460780.

IV. *Applicability of supercritical fluid chromatography – mass spectrometry to metabolomics. II – Assessment of a comprehensive library of metabolites and evaluation of biological matrices*.
G.L. Losacco, O. Ismail, J. Pezzatti, V. González-Ruiz, J. Boccard, S. Rudaz, J.-L. Veuthey, D. Guillarme; J. Chromatogr. A, 1620 (2020), 461021.

V. *Investigating the use of unconventional temperatures in supercritical fluid chromatography.*
G.L. Losacco, S. Fekete, J.-L. Veuthey, D. Guillarme, Anal Chim Acta, 1134 (2020), 84-95.

VI. *Ultra-high performance supercritical fluid chromatography coupled to tandem mass spectrometry for antidoping analyses: assessment of the inter-laboratory reproducibility with urine samples.*
G.L. Losacco, M. Rentsch, K. Plachka, F. Monteau, E. Bichon, B. Le Bizec, L. Novakova, R. Nicoli, T. Kuuranne, J.-L. Veuthey, D. Guillarme, Analytical Science Advances (2020), https://doi.org/10.1002/ansa.202000131

VII. *Expanding the range of sub/supercritical fluid chromatography: advantageous use of methanesulfonic acid in water-rich modifiers for peptide analysis.*
G.L. Losacco, J.O. DaSilva, J. Liu, E.L. Regalado, J.-L. Veuthey, D. Guillarme; J. Chromatogr. A, 1642 (2021), 462048.

VIII. *Metamorphosis of supercritical fluid chromatography: is it now a viable tool for the analysis of polar substances?*
G.L. Losacco, J.-L. Veuthey, D. Guillarme; Trends Anal. Chem. 141 (2021), 116304.

Other scientific articles

I. *Enantiomeric methadone quantification on real post-mortem dried matrix spot samples: comparison of liquid chromatography and supercritical fluid chromatography to mass spectrometry.*
F. Müller, G.L. Losacco, R. Nicoli, D. Guillarme, A. Thomas, E. Grata; J. Chromatogr. B, 1177 (2021), 122755.

II. *Using 1.5 mm internal diameter columns for optimal compatibility with current liquid chromatographic systems*
S. Fekete, A. Murisier, G.L. Losacco, J. Lawhorn, J.M. Godinho, H. Ritchie, B.E. Boyes, D. Guillarme; J. Chromatogr. A, 1650 (2021), 462258.

III. *Interlaboratory study of a Supercritical Fluid Chromatography method for the determination of pharmaceutical impurities: evaluation of multi-systems reproducibility.*
A. Dispas, A. Clarke, A. Grand-Guillaume Perrenoud, G.L. Losacco *et al*; J. Pharm. Biomed. Anal., 203 (2021), 114206.

Scientific articles under preparation

I. *Chapter 2 – Application space for SFC in pharmaceutical drug discovery and development.*
G.L. Losacco, A. Dispas, J.-L. Veuthey, D. Guillarme; Book chapter in “Practical Application of Supercritical Fluid Chromatography for Pharmaceutical Research and Development” (submitted - 2021).

Oral communications

I. *SFC-MS and metabolomics: a possible marriage?*
J.L. Veuthey, G.L. Losacco, S. Rudaz, D. Guillarme; 48th International Symposium on High-Performance Liquid Phase Separations and Related Techniques (HPLC 2019); June 2019 - Milan (Italy).

II. *Is SFC-MS a viable tool in doping control analyses?*
G.L. Losacco, R. Nicoli, T. Kuuranne, J.-L. Veuthey, D. Guillarme; RDPA 2019; September 2019 - Pescara (Italy).

III. *Expanding the applicability of SFC-MS in the pharmaceutical and forensic environment.*
G.L. Losacco; 33rd Seminar in Pharmaceutical Sciences; September 2019 - Zermatt (Switzerland).

IV. *Can SFC-MS/MS be considered a viable alternative to LC-MS/MS in the routine control analysis of prohibited substances in urine?*
G.L. Losacco, R. Nicoli, T. Kuuranne, J.-L. Veuthey, D. Guillarme; SFC 2019; October 2019 - Philadelphia (USA).

V. *La chromatographie en phase supercritique pour les analyses antidopage de routine : rêve ou réalité ?*
G.L. Losacco, Journée Scientifique du Club AFSEP lyonnais, May 2021, Lyon (France).

Poster communications

I. *SFC-MS in metabolomics: Optimization of the mobile phase conditions for the simultaneous analysis of hydrophilic and lipophilic substances.*
G.L. Losacco, S. Rudaz, J.L. Veuthey, D. Guillarme; 12th International Conference on Packed-Column SFC (SFC 2018); October 2018 – Strasbourg (France).

II. *Application de la SFC-MS à la mètabolomique : optimisation des conditions de la phase mobile pour l'analyse simultanée des substances hydrophiles et lipophiles.*
G.L. Losacco, S. Rudaz, J.L. Veuthey, D. Guillarme; 14th Congrès francophone de l'AFSEP sur les Sciences Séparatives et le Couplages (SEP 2019); March 2019 – Paris (France).

Abbreviation list

ACN	Acetonitrile
AmAc	Ammonium Acetate
AmF	Ammonium Formate
APCI	Atmospheric Pressure Chemical Ionization
API	Atmospheric Pressure Ionization
APPI	Atmospheric Pressure Photo Ionization
BEH	Bridge Ethylene Hybrid
BPR	Back Pressure Regulator
CI	Chemical Ionization
CID	Collision Induced Dissociation
CO_2	Carbon Dioxide
C18	Octadecyl alkyl selector
DCM	Dichloromethane
DEA	Diethylamino column
DS	Dilute-and-shoot
EFLC	Enhanced Fluidity Liquid Chromatography
EI	Electron Impact
ESI	Electrospray Ionization
EtOH	Ethanol
FPP	Fully Porous Particle
FWHM	Full Width at Half Maximum
GC	Gas Chromatography
H	HETP = height equivalent to theoretical plate
HILIC	Hydrophilic Interaction Chromatography
HRMS	High resolution mass spectrometry
HSS	High Strength Silica
iPrOH	Isopropanol
IT	Ion Trap
k	Retention factor
LC	Liquid Chromatography
LLE	Liquid-Liquid Extraction

LSER	Linear Solvent Energy Relationship
m/z	Mass over charge ratio
ME	Matrix Effect
MeOH	Methanol
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
MSA	Methanesulfonic Acid
MTBE	Methyl Ter-Butyl Ether
MW	Molecular Weight
NPLC	Normal Phase Liquid Chromatography
pI	Isoelectric point
PP	Protein Precipitation
ppm	Part per million
Q	Quadrupole
QC	Quality Control
QqQ	Triple quadrupole
QqTOF	Tandem quadrupole – time of flight
RPLC	Reversed Phase Liquid Chromatography
S/N	Signal to Noise
scCO2	Supercritical Carbon Dioxide
SEF	Silyl Ether Formation
SFC	Supercritical Fluid Chromatography
SFE	Supercritical Fluid Extraction
SIM	Single Ion Monitoring
SPE	Solid Phase Extraction
SPP	Superficially Porous Particle
SRM	Single Reaction Monitoring
TFA	Trifluoroacetic Acid
TOF	Time of Flight
u	Linear Velocity
UC	Unified Chromatography
UHPLC	Ultra-High Performance Liquid Chromatography
UHPSFC	Ultra-High Performance Supercritical Fluid Chromatography
UPC^2	Ultra Performance Convergence Chromatography

UV	Ultra-Violet
WADA	World Anti-Doping Agency
2-PIC	2-Picolylamine

Chapter I – Introduction

I.1 Interest of polar compounds and their analysis

Compounds can be classified in different ways according to their physical-chemical properties. One of the most important parameters is polarity. A molecule is defined as polar when its total dipolar moment (μ) is different from zero. Several functional groups can give a polar character to a compound, such as alcohols (-OH), aldehydes (-CHO), carboxylic acids (-COOH) and amines ($-NR_2$). Their presence provides a high polarity degree to a molecule, thus improving its solubility in polar solvents (e.g., water) thanks also to different interactions generated, such as H-bonding and dipole-dipole among others. On the contrary, functional groups such as alkyl or aromatic rings decrease the polarity of a molecule. A parameter commonly used to describe the lipophilicity or hydrophilicity of a compound is the *log P* value. It represents the partition coefficient of the analyte between the organic (apolar) phase and the aqueous (polar) phase, as described in Equation 1:

$$\log P = \log \frac{C\ octanol}{C\ water} \quad (1)$$

$C_{octanol}$ is the concentration of the analyte in octanol and C_{water} is its concentration in water. High *log P* (*log P* > 0) values correspond to a balance in favor of the molecule hydrophobicity, while negative *log P* values (*log P* < 0) indicate a higher polarity. Another parameter widely employed is the *log D*, which takes into consideration all the forms of the molecule (neutral and ionic forms). Therefore, log D strongly depends on the aqueous phase's pH as well as the pKa value of the analyte.

Polar compounds can be found in many domains, from the pharmaceutical industry to forensic analyses, as well as metabolomics. Polar metabolites are widespread in the human organism, contributing to almost all biological processes. Compounds of significant pharmaceutical interest such as amino acids, peptides, nucleosides, and nucleotides are also highly polar, as well as their metabolites. Some substances which are illegal in many domains, for example doping agents in sports, also possess a relatively high polarity. To perform analyses on such compounds, researchers soon realized that reversed phase liquid chromatography (RPLC) is not the best analytical technique, as it separates analytes mostly based on their hydrophobicity. Other approaches, such as hydrophilic interaction chromatography (HILIC) or capillary electrophoresis (CE) can be successfully used, but present different issues such as long time analysis or poor reproducibility. Therefore, new strategies are currently under development for the analysis of highly polar compounds.

I.2 Recent development in UHPSFC

SFC underwent an impressive evolution in its applicability range in the past 5-10 years. Latest trends indicate how this technique seems to have become not only an alternative to NPLC and RPLC analyses, but also to HILIC for the analysis of highly polar compounds, as well as samples with an increasing molecular weight.

In this section, an overview of the instrumentation available on the market, as well as a brief description of the stationary phases conventionally used is given. In addition, the evolution of the mobile phase composition throughout the years is also discussed. Finally, in the last part of this section, a review article describing the latest trends in SFC analysis is provided.

I.2.1 UHPSFC instrumentation and stationary phases

SFC is a technique known since several years, despite that it did not manage to establish itself in analytical laboratories until recently. Originally conceived with the pioneering work of Klesper *et al.* in the early 1960s (1), SFC experienced several moments of fame, as well as unpopularity throughout its history. It became almost immediately abandoned in the 1960s and 1970s, as GC and LC became the main focus of all scientists involved in separation sciences. However, starting from the 1980s, interest arose on this technique once more. The instrumentation available at the time was able to reach and maintain the supercritical state of some compounds, thus enabling SFC analysis. To obtain such state, it is mandatory to be at the critical point set for a given compound, defined by specific critical pressure and temperature values. Carbon dioxide's critical point is found at approximately 73.8 bar (7.38 MPa) and 31.10°C (304.13°K), as described in Figure 1.

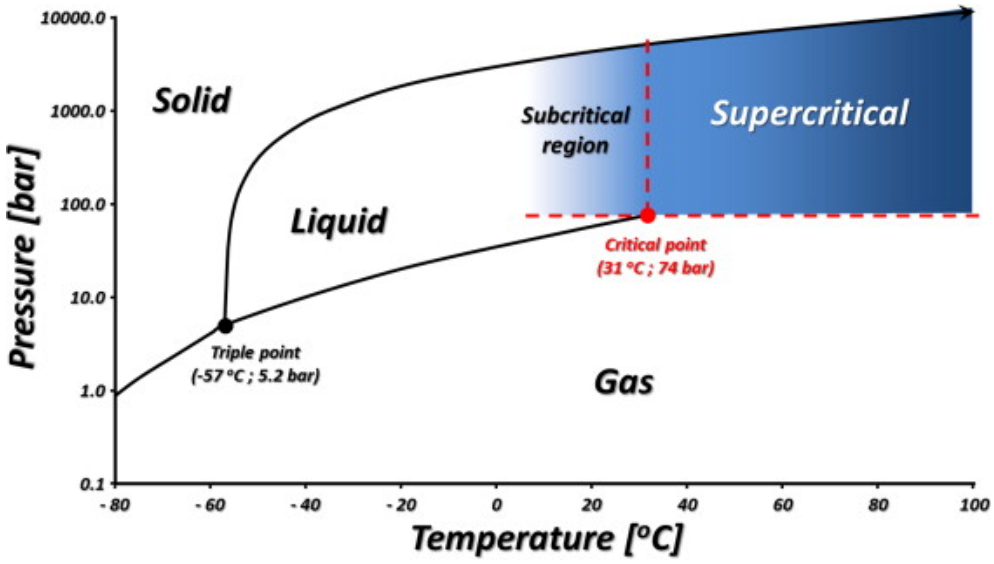

Figure 1: Phase diagram of carbon dioxide. Reprinted from (2).

Therefore, initial applications of SFC involved a mobile phase composed of pure supercritical CO_2. Unfortunately, due to the low polarity of $scCO_2$, which was found to be close to that of organic solvents such as *n*-hexane or *n*-heptane, it was only possible to analyze apolar substances such as lipids or hydrocarbons (3,4). The need to expand the applicability range of SFC translated into the development of new instruments that were able to add a liquid modifier to the $scCO_2$. While the presence of the co-solvent greatly improved the range of applications of SFC, especially towards compounds of pharmaceutical interest such as synthetic drugs (5,6), the instrumentation was plagued with different issues. SFC was, in general, not able to guarantee the needed quantitative performance (precision, robustness, etc.) present in GMP laboratories. These problems were due to the poor handling of the supercritical mobile phase, which is heavily subjected to density shifts that would, in the end, generate important retention shifts in SFC (7). Moreover, the UV sensitivity of this technique was much lower than that of LC, as a much larger background noise was generally visible in SFC (7,8).

Because of the aforementioned issues, SFC was often overlooked as a niche technique valid only for chiral analyses, with its undeniable advantages obscured by the many instrumentational problems. This situation drastically changed in the early 2010s, as a new and improved generation of SFC systems was released on the market by different instrument providers, such as Waters (Milford, USA) with the Ultra-Performance Convergence Chromatography (UPC^2), Agilent Technologies (Waldbronn, Germany) with the 1260 Infinity I and II Hybrid SFC/UHPLC systems, and also Shimadzu (Kyoto, Japan) with the Nexera Unified Chromatography instrument. Although different instrument developers have released their version of the SFC system, they all possess the same configuration (Fig. 2). More importantly, however, their development allowed SFC to perform an incredible step forward in terms of performance, from a kinetic point of view, but also in terms of method robustness and sensitivity (2,9). They also allowed the implementation of stationary phases packed with sub-2 µm particles. Therefore, SFC experienced a similar transition observed from HPLC to UHPLC; similarly, the term Ultra-High Performance SFC (or UHPSFC) began appearing in the literature. A more detailed description of the different SFC systems can be found in the thesis works of Dr. Alexandre Grand-Guillaume Perrenoud (University of Geneva, Thesis n°4717) and Dr. Vincent Desfontaine (University of Geneva, Thesis n°5181).

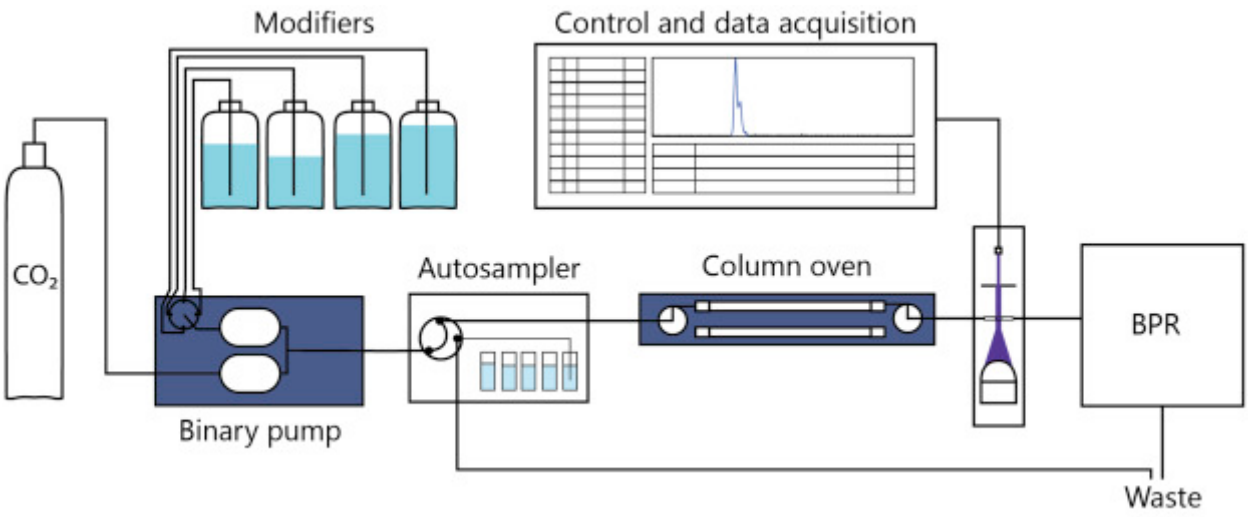

Figure 2: Illustration of a generic UHPSFC system. Reprinted from (10).

I.2.2 Stationary phases

Stationary phases used nowadays in SFC are based on silica particles having different diameters, mainly 3.0 µm, 2.5 µm or sub-2 µm fully porous particles, as well as sub-3 µm superficially porous particles. Specifically, the sub-2 µm FPP, similarly to what was observed for LC in the early 2000s, have been responsible in an incredible boost in terms of performance achievable in SFC, and with its transformation into UHPSFC (11). In addition to LC, however, it is possible with SFC to reach high flow-rates without experiencing unacceptable backpressures. This is due to the much lower viscosity of the SFC mobile phase compared to the LC one. As a result, the minimum height of the theoretical plates (H) given by the van Deemter equation (Equation 2) that can be reached in SFC is shifted towards the C-term region of the van Deemter curve, compared to what can be obtained under LC conditions.

$$H = A + \frac{B}{u} + C * u \qquad (2)$$

where *H* corresponds to the plate height, *u* is the mobile phase linear velocity, and *A*, *B* and *C* are the coefficients of the van Deemter equation. This means that similar, or even better kinetic performance can be obtained in SFC at higher velocities (Fig. 3).

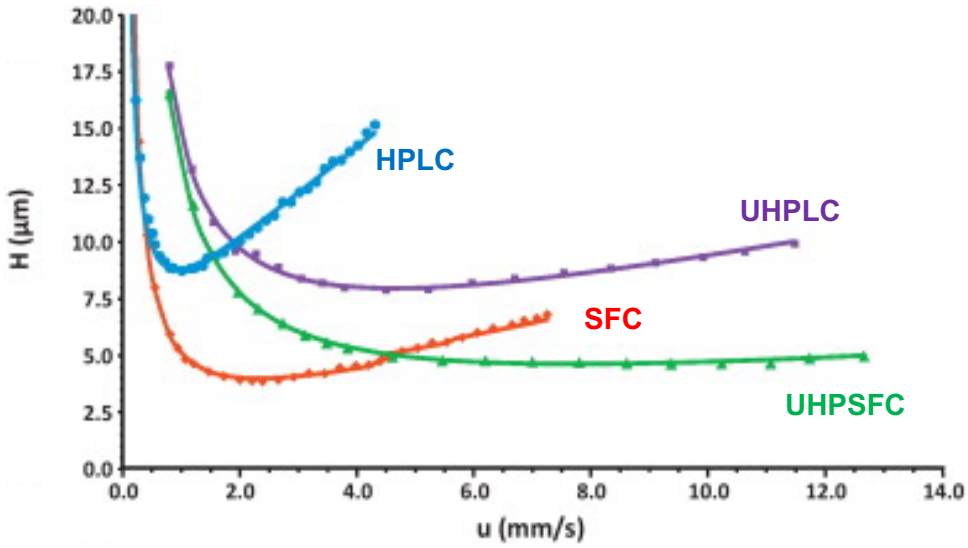

Figure 3: van Deemter curves for butylparaben on two systems equipped with 1.7 or 3.5 µm particles columns. XTerra RP18 50 mm × 4.6 mm, 3.5 µm (blue dots), Acquity Shield C18 50 mm × 2.1 mm, 1.7 µm (red diamonds) both columns tested in LC conditions. Acquity UPC^2 BEH 2-EP 100 mm × 3.0 mm, 3.5 µm (purple squares) and 100 mm × 3.0 mm, 1.7 µm (green triangles) columns tested in SFC conditions. Adapted, with permission, from (12).

Regardless of the silica particle size, stationary phases in SFC present a somewhat more important role than in LC. This is due to the peculiar retention mechanism involved in SFC-based separations. Indeed, contrary to LC techniques such as RPLC, NPLC or HILIC, in which analytes are retained either based on their hydrophobicity (RPLC), or mainly on their polarity (NPLC and HILIC), in SFC, compounds interact with the stationary phase via multiple interactions. H-bonds, as well as dipole-dipole, ionic and π-π interactions can be formed in SFC. Hence, it becomes clear that SFC could provide a higher degree of flexibility. Different stationary phases previously developed for LC have been used in SFC: NPLC columns were largely employed among the first applications of packed SFC, making the latter an alternative for chiral analysis (8,13,14) and, to a lesser extent, for achiral separations of lipophilic and moderately polar analytes (3,15). From the 2000s, column manufacturers began the development and subsequent release on the market of the first stationary phases dedicated to SFC. These columns were polar but some modifications were made to improve the peak shape with specific categories of analytes, such as basic compounds (16,17). With their use, SFC demonstrated good performance also for RPLC achiral analyses, showing a high degree of orthogonality (2,18).

However, it was only in the past decade that SFC was capable to demonstrate its real potential. As a new generation of UHPSFC stationary phases was developed by Waters with the Acquity Torus columns, followed by other column manufacturers, the applications involving SFC exponentially grew. However, as the number of columns potentially used in SFC has become

quite large, a classification based on the linear solvation energy relationships (LSER) with Abraham descriptors has been developed and constantly updated by the group of Lesellier and West (19) (Fig.4).

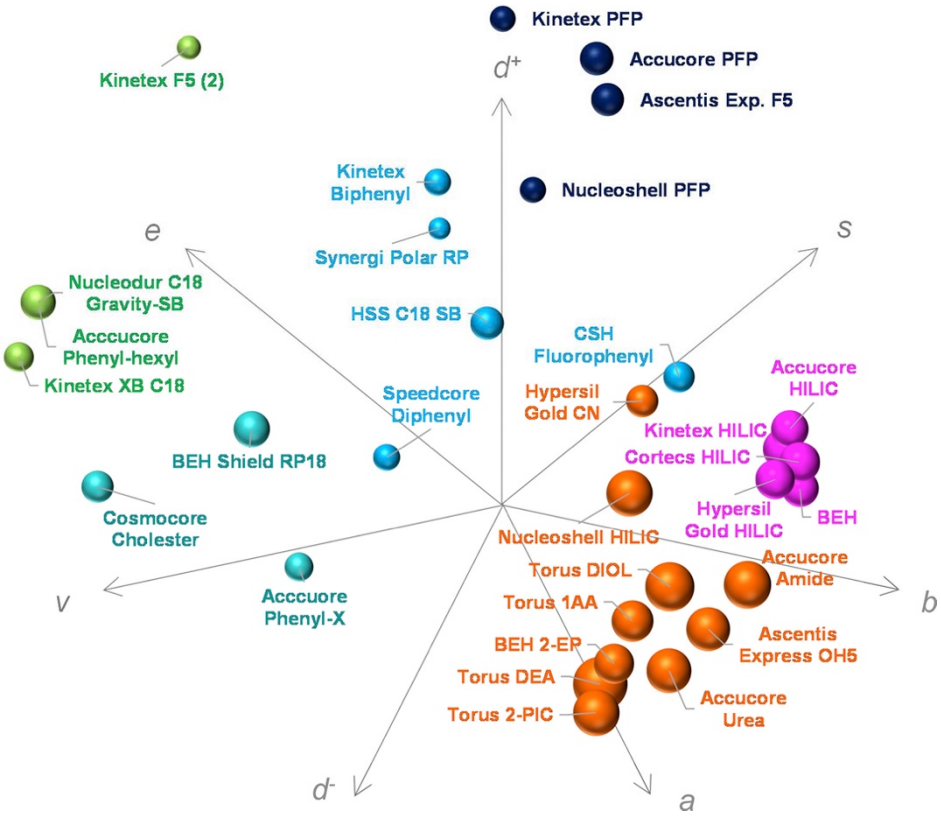

Figure 4: Spider diagram based on the LSER models calculated with 7 descriptors. Reprinted from (19)

As discussed in the review article in section I.1.5, a gradual shift towards the analysis of compounds of increasing polarity and complexity was possible thanks to the use of innovative stationary phases. Today, SFC has the potential to cover an impressive range of analytes with extremely different polarity using a single technique, in opposition to LC for which multiple modes are required. Furthermore, the development of new UHPSFC stationary phases also provided a reduced presence of free silanols on the surface of the silica particles (19). This, in combination with the systematic addition of water in the mobile phase, has greatly contributed to guarantee a satisfactory robustness and low variability of retention times using SFC-based methods (20). In conclusion, the availability of numerous stationary phase chemistries and morphologies provides to SFC the necessary performance to compete with LC techniques, guaranteeing the possibility to obtain a fine-tuning of the separation.

I.2.3 Role of the modifier and additives

The SFC mobile phase has been the subject of several works from different research groups involved in the development of this technique. Due to its peculiar nature (it is composed of a mixture of a supercritical fluid with a liquid modifier), it worked as a double-edged sword. On one hand, the possibility to mix an apolar solvent ($scCO_2$) with a polar modifier (MeOH, EtOH, iPrOH, etc.) and the use of different additives (water, TFA, formic acid, ammonia, alkylamines, AmF, AmAc, etc.) at different ratios, in combination with different column chemistries, gives an unprecedented flexibility to chemists in obtaining the best separation possible for an increasing pool of compounds. On the other hand, however, it makes the entire method development process more complicated, as different combinations need to be explored. Moreover, the mobile phase undergoes a state change when the modifier percentage increases. The transition from a supercritical to a subcritical fluid is observed with co-solvent percentages higher than 10-15% (7). The mobile phase would continue evolving into a liquid-like solvent as ratios of $scCO_2$ to modifier would progressively change to 70/30 *v/v*. As the liquid component becomes more predominant, a more liquid-like behavior of the mobile phase was noticed (21). This translated mainly into worsening strong reduction of the kinetic performance with the older generations of SFC instruments. Fortunately, thanks to the reduced system volumes and much improved pump designs of UHPSFC systems, these issues have been addressed to a great extent. This enable researchers to be more innovative and, as a consequence, to push the elution limits of SFC. As described in detail in the review article of section I.2.4, the implementation of new gradient profiles reaching modifier percentages as high as 100% are being more and more utilized in the method development process for highly polar substances, such as nucleosides, sugars, polar metabolites or peptides.

As mentioned in the beginning of this section, SFC mobile phases are based on the mixture of $scCO_2$ with a liquid modifier at different percentages. Regardless of the ratio between these two components, a key role is played by the nature of the co-solvent utilized. This has been subjected to an incredible number of changes throughout the course of SFC history. While different solvents, miscible with the supercritical CO_2, have been tested with various success especially during the 1980s and early 1990s, the choice soon fell on alcohols with small alkyl chain (22,23). Methanol, ethanol and isopropanol became the most common modifiers, with MeOH in particular being the favorite choice, especially in the context of achiral separations. Their addition to $scCO_2$ ensures the needed increase in the mobile phase's elution strength, thus enabling the analysis of compounds with a moderate polarity ($\log P \sim 0$). However, this was not sufficient if more polar substances have to be analyzed. Immediately, researchers began experimenting the use of additives in the modifier. Acidic compounds, such as TFA or

formic acid, as well as basic molecules (diethyl- and triethylamine, ammonia) were employed at relatively low percentages (< 0.2%) in the modifier (24). Later on, ammonium salts such as AmF or AmAc have been added at low concentrations (< 10 mM) (9). The chromatographic performance benefited from an impressive boost, with much sharper peaks and improved peak symmetry. This is due to different mechanisms, such as ion-pairing phenomenon, increased polarity of the mobile phase, as well as adsorption onto the stationary phase. Changes in the mobile phase apparent pH can also be observed, however in a recent article (25) this seems to be true only with strong acids such as TFA (Fig. 5). This seems to be caused by the *in situ* formation of carbonic and methyl-carbonic acid between CO_2, MeOH.

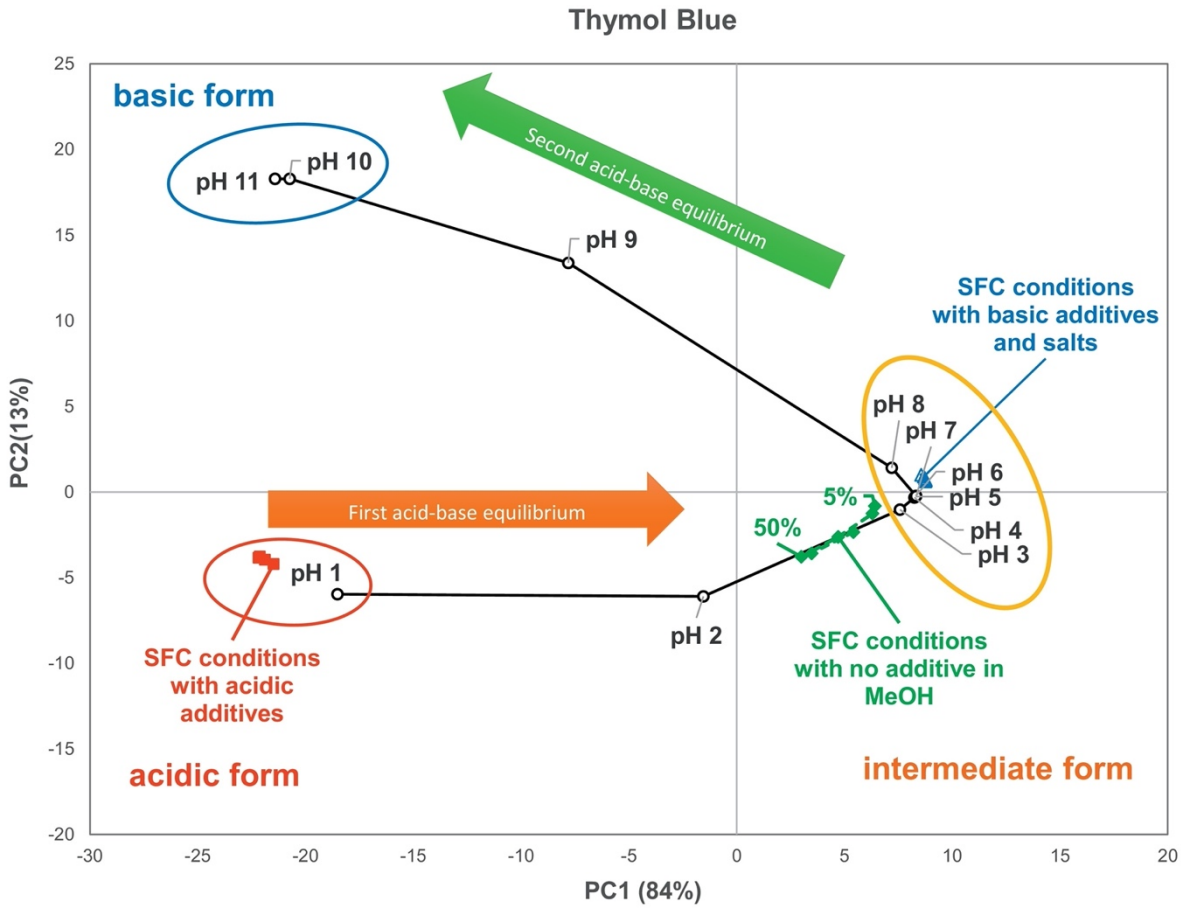

Figure 5: PCA graph indicating pH shifts in liquid buffered solvent and in SFC mobile phase. Blue triangles: basic additives (diethylamine, isopropylamine, diethanolamine) and salts (ammonium hydroxide and ammonium acetate), all 20 mM. Red squares: acidic additives (acetic acid, formic acid, trifluoroacetic acid, methanesulfonic acid, all 0.1%) or water 2%. Reprinted, with permission, from (25).

Among the different tested additives, water started to attract more attention among analysts. Its use at relatively low percentages (up to 5-7% in the liquid co-solvent) revealed to be fundamental to ensure good chromatographic performance of SFC in various fields, from metabolomics to peptide analysis (26,27). Finally, it contributed to reduce the precipitation

phenomenon of different analytes, and also offer the possibility to employ higher concentrations of additives in the mobile phase. More details on how water benefits SFC-based separations are given in the review article in section I.2.4.

I.2.4 Review article: UHPSFC for the analysis of polar compounds

In the following review article, published in 2021, an outline of how SFC has evolved into a technique suitable for the analysis of highly polar compounds is given. Examples of the latest applications developed with this technique are also highlighted.

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Metamorphosis of supercritical fluid chromatography: A viable tool for the analysis of polar compounds?

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ABSTRACT

Recent developments in supercritical fluid chromatography (SFC) have highlighted the applicability of this technique for the analysis of highly polar compounds. The combination of polar stationary phases and CO_2-based mobile phases with an increasing presence of liquid co-solvent (up to 100%) has enabled to further expand the application field of SFC towards a variety of samples such as polar endogenous metabolites, plant extracts, water-soluble vitamins, pesticides, sugars, peptides and so on. In this evolution, a key role was played by the addition of up to 5–10% of water in the liquid co-solvent. Moreover, the presence of water enabled higher concentrations of additives, up to 75–100 mM in some cases. These improved conditions were fundamental in expanding the applicability range of SFC. Overall, SFC has demonstrated its evolution into a mature technique capable of offering a true alternative to liquid chromatography for the analysis of polar compounds.

1. Introduction

Analytical laboratories are involved in a constant effort to develop their workflow, as they are facing with the need to analyze increasingly complex samples. In the pharmaceutical environment, scientists are often required to perform assays on compounds showing quite different physical-chemical properties (e.g. size, lipophilicity, ionizable groups …), going from small synthetic drugs to peptides and proteins [1–3]. Obviously, as the samples of interest show such different characteristics, the analytical technique has to be adapted. Liquid chromatography (LC) is highly versatile and has risen as one of the most employed approaches to analyze a wide variety of compounds [3]. Thanks to its various modes of operation, LC can successfully guarantee the analysis of samples covering log P values ranging from 10 to −10 (Fig. 1). Therefore, lipids, steroids, synthetic drugs, nucleosides, sugars, peptides and proteins have been successfully analyzed by using the LC mode that fits best to each analyte category [4–7].

While LC can guarantee a suitable retention for an impressive range of samples, it also shows some drawbacks. Normal phase LC (NPLC) employs toxic solvents (e.g. hexane, dichloromethane, etc.), therefore it is not considered environmentally friendly [8,9]. Reversed phase LC (RPLC) shows an excellent robustness and ease of transferability among different laboratories, but it is unsuitable to analyze highly polar substances [10,11]. Hydrophilic interaction chromatography (HILIC) was developed to better retain the latter, but long equilibration times are often needed [10]. Moreover, both techniques employ large amount of organic solvents, thus they cannot be considered as green analytical strategies [12]. Therefore, analysts have begun to shift their interest towards alternative solutions. Among them, supercritical fluid chromatography (SFC) and its modern version known as ultra-high performance supercritical fluid chromatography (UHPSFC) could be attractive [13,14]. UHPSFC employs a mixture of supercritical carbon dioxide ($scCO_2$) mixed with polar organic modifier, in most cases methanol (MeOH), using columns packed with sub-2μm particles. As highlighted in Fig. 1, UHPSFC can be considered a valid alternative to NPLC and RPLC for apolar and mildly polar substances [14]. UHPSFC methods have been generally developed following some specific guidelines. As an example, pure methanol was often chosen as the liquid co-solvent, especially when samples with limited polarity were analyzed. Gradient profiles with a maximum percentage of approximately 35–40% modifier mixed with supercritical CO_2 were constantly used. However, many analysts have recently begun to evaluate UHPSFC for hydrophilic analytes such as polar metabolites or water-soluble vitamins. In 2014, a work published by Taguchi et al.

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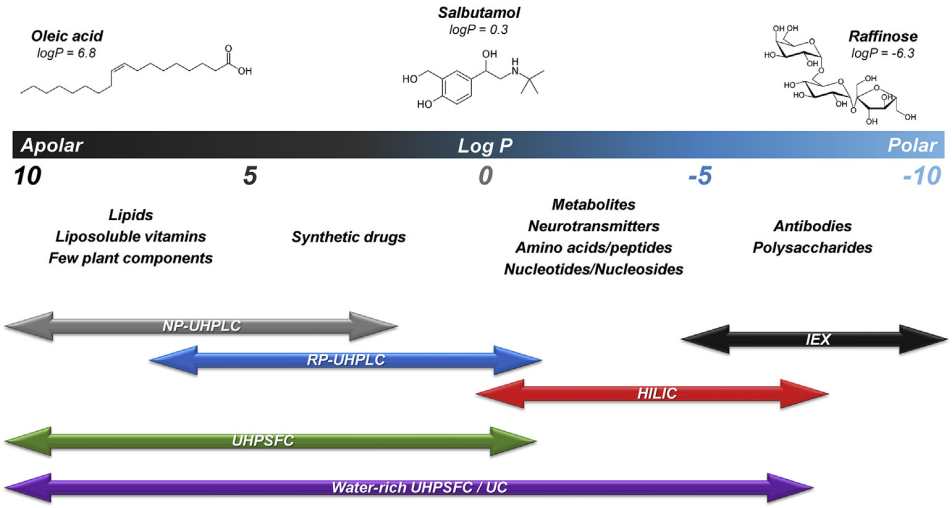

Fig. 1. Polarity range covered by different LC techniques (NPLC, RPLC, HILIC and IEX), compared to UHPSFC and water-rich UHPSFC/UC.

illustrated how the combined use of a water-rich modifier with a gradient profile up to 100% in the organic modifier, also known as "Unified Chromatography" (UC), enabled the simultaneous analysis of water soluble and liposoluble vitamins in UHPSFC [15]. Hence, the use of water-rich UHPSFC or UC conditions can potentially expand the polarity range of UHPSFC, allowing its use as an alternative not only to NPLC and RPLC, but also to HILIC (Fig. 1).

The aim of this review is to analyze and describe the latest trends in UHPSFC, focusing on its potential implementation with highly polar substances. A description of how UHPSFC mobile phase has evolved to guarantee the elution of such compounds is given, as well as on different gradient profiles which have arisen in recent years. Afterwards, a focus on the choice of the stationary phase is provided, highlighting the possible advantages of UHPSFC over UHPLC, as well as potential drawbacks and limitations. Finally, a detailed overview of the latest applications developed for a variety of compounds such as polar biomolecules, plant extracts, polar metabolites and pesticides is given, focusing also on how UHPSFC has evolved in a potential choice for multidimensional systems coupled to UHPLC techniques.

2. Evolution of UHPSFC mobile phase

2.1. Use of water-rich organic modifier

The implementation of water in SFC is not a new trend. Research groups initially tested water as an alternative to polar small alcohols to increase the polarity of $scCO_2$-predominant mobile phase [16]. However, its poor miscibility with supercritical CO_2 discouraged analysts to employ it as a pure modifier, but rather in combination with organic solvents such as methanol or ethanol [17,18]. In recent years, the use of water-rich modifier has started to become more preponderant. Various percentages of water, from 1% up to 7–8% mixed with methanol, have been used in UHPSFC methods for the analysis of various substances, such as polar metabolites [19–23], doping agents [20,24–26], plant extracts [27–29] as well as peptides and proteins [30–32]. Water became an increasingly employed additive also for chiral UHPSFC separations [33–36]. Applications involving UHPSFC to analyze strongly retained substances on the polar stationary phase, such as hydrophilic analytes, was sometimes difficult to develop in the past. Indeed, the "standard" choice of the organic modifier was often based on the use of pure methanol [37–39]. Such co-solvent, however, could not guarantee a sufficient eluent strength for the UHPSFC mobile phase, as the compounds are too strongly retained by the stationary phase. Therefore, either long time analysis or, in the worst cases, no elution at all of highly polar substances is observed [33,34]. The use of water in the organic modifier has enabled a remarkable increase in the elution strength of the UHPSFC mobile phase. As the extension of the gradient profile increases the presence of the modifier, and therefore water, a stronger competition between the stationary and mobile phases for the sample arises [34,40]. Consequently, a reduction of analysis time can be observed in most cases (Fig. 2), and the elution of strongly retained compounds becomes possible [34,35]. Another benefit to add water in the SFC mobile phase is to obtain sharper and more symmetrical peak shapes [28,41,42] (Fig. 2). Furthermore, the use of water as a simple additive greatly improves the reproducibility of UHPSFC analytical methods in some cases. It was indeed recently demonstrated how its systematic use contributed to generate low retention times variability for a set of doping agents in biological matrices across an extended timeframe (up to four months), as well as to reduce the inter-laboratory variability for the same UHPSFC method [24,25]. The authors claimed that the constant exposure of the stationary phase to water helped minimizing the formation of methyl-silyl ethers on the free silanols, a phenomenon which was proved to be detrimental in UHPSFC causing potential changes in the elution profile [43].

2.2. Use of high concentration of additives

The systematic implementation of water in the organic modifier was not the only development concerning UHPSFC mobile phases in recent years. Another feature allowing the analysis of highly polar molecules in UHPSFC has been the use of additives, such as trifluoroacetic acid (TFA), ammonia, ammonium formate (AmF) and acetate (AmAc), at relatively high concentrations (equal or above 20 mM) [42,44,45]. While acidic and basic additives, available under their liquid form, never presented miscibility issues regardless of their concentration, there was a limit in the concentration reachable for AmF and AmAc in the organic modifier, when using pure methanol. The addition of water to MeOH ensured, as expected, a much-improved solubility of these salts, thus allowing their use at higher concentrations. As demonstrated in recent

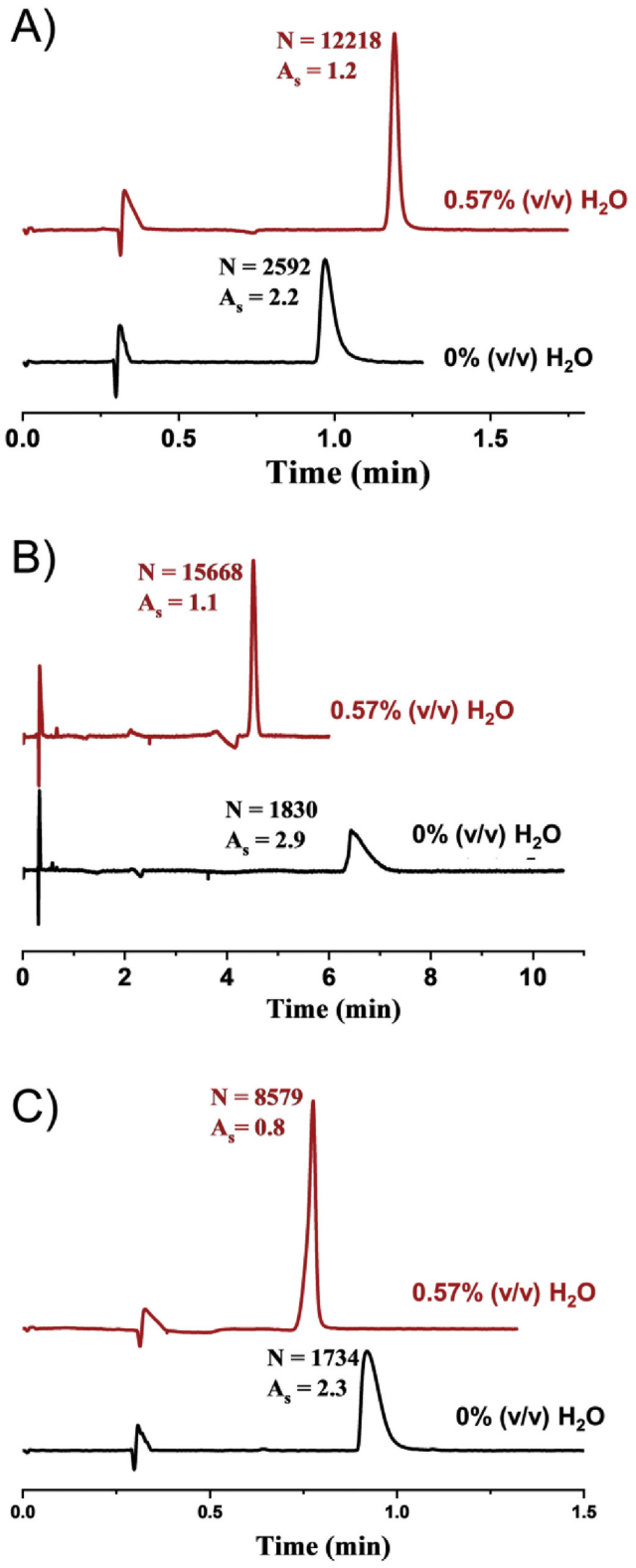

Fig. 2. SFC chromatograms of a) nicotinic acid, b) 1-methyl-3-phenylpropylamine and c) 3,5-dinitrobenzoic acid on FructoShell-N column with or without the addition of water in the mobile phase. Reprinted with permission from Ref. [34].

articles, levels up to 150 mM of salts were reached in the organic modifier (Fig. 3) thanks to the presence of water [23,45,46], while in one work it was possible to use concentrations exceeding 1 M of liquid additives in pure methanol [47]. As shown in these papers, a high amount of additives provided a significant improvement of chromatographic parameters such as peak shapes and peak widths (Fig. 3). However, using concentrations as high as 150 mM could present issues when coupling UHPSFC with mass spectrometry (MS) [23]. Issues such as low MS sensitivity, as well as a higher risk of salt precipitation in the mobile phase might still occur [48]. Nonetheless, concentrations of buffers ranging from 20 to 50 mM have been used to analyze a series of challenging analytes such as amino acids [22,45,46], biosurfactants [49] and a series of polar analytes (log P < −2) present in environmental water samples [50].

As previously mentioned, the choice of the additive has been limited due to potential solubility issues in the $scCO_2$/MeOH mobile phase. However, as water is present in the mobile phase, it enabled either the use of salts which are poorly soluble in organic solvents [23] or the combination of multiple additives in the organic modifier [22,26], due to its improved solvation capability.

2.3. Choice of the gradient profile

Researchers have also focused their efforts on the use of an unconventional ratio of $scCO_2$ to organic modifier, with the goal to further increase the elution strength of $scCO_2$-based mobile phases. One of the potential solutions is the use of innovative gradient profile called “Enhanced Fluidity Liquid Chromatography” (EFLC). With EFLC-type gradients, the amount of $scCO_2$ is reduced and does not reach percentages above 35–40% in the mobile phase. In some of the latest applications involving this technique, it was even demonstrated how an increased presence of water, mixed with the supercritical CO_2, was possible [51,52]. CO_2 is, therefore, used mainly to reduce the mobile phase viscosity. By doing so, the advantages typically observed with the use of $scCO_2$-predominant mobile phases are still observed (i.e. enhanced kinetic performance, reasonable backpressure), while miscibility issues between all components of the mobile phase are reduced [53]. With this approach, it was possible to perform analyses on polysaccharides [53], as well as proteins [52].

Another approach developed by research groups is the “Unified Chromatography” (UC) gradient [15]. The idea with UC-type gradients is quite simple: to ensure the successful elution of the most retained compounds from the stationary phase, the amount of polar co-solvent can be increased, to improve the elution strength, thus ensuring that even highly-retained analytes can be eluted and detected [22,45]. The organic modifier employed in UC-type gradients should not reach high amounts of water, in order to ensure its full miscibility with the $scCO_2$. The advantage of such gradient would be the possibility to perform simultaneous analyses of lipophilic and hydrophilic substances. In one of the first UC gradient application, UHPSFC demonstrated good performance analyzing, at the same time, lipophilic and hydrophilic vitamins [15]. UHPSFC was also successfully used to analyze apolar and polar endogenous metabolites spiked in biological matrices [22].

3. UHPSFC stationary phases

3.1. Impact of high modifier percentages on kinetic performance

The use of EFLC or UC-based gradients comes, however, with different constraints and issues. As the liquid component increases,

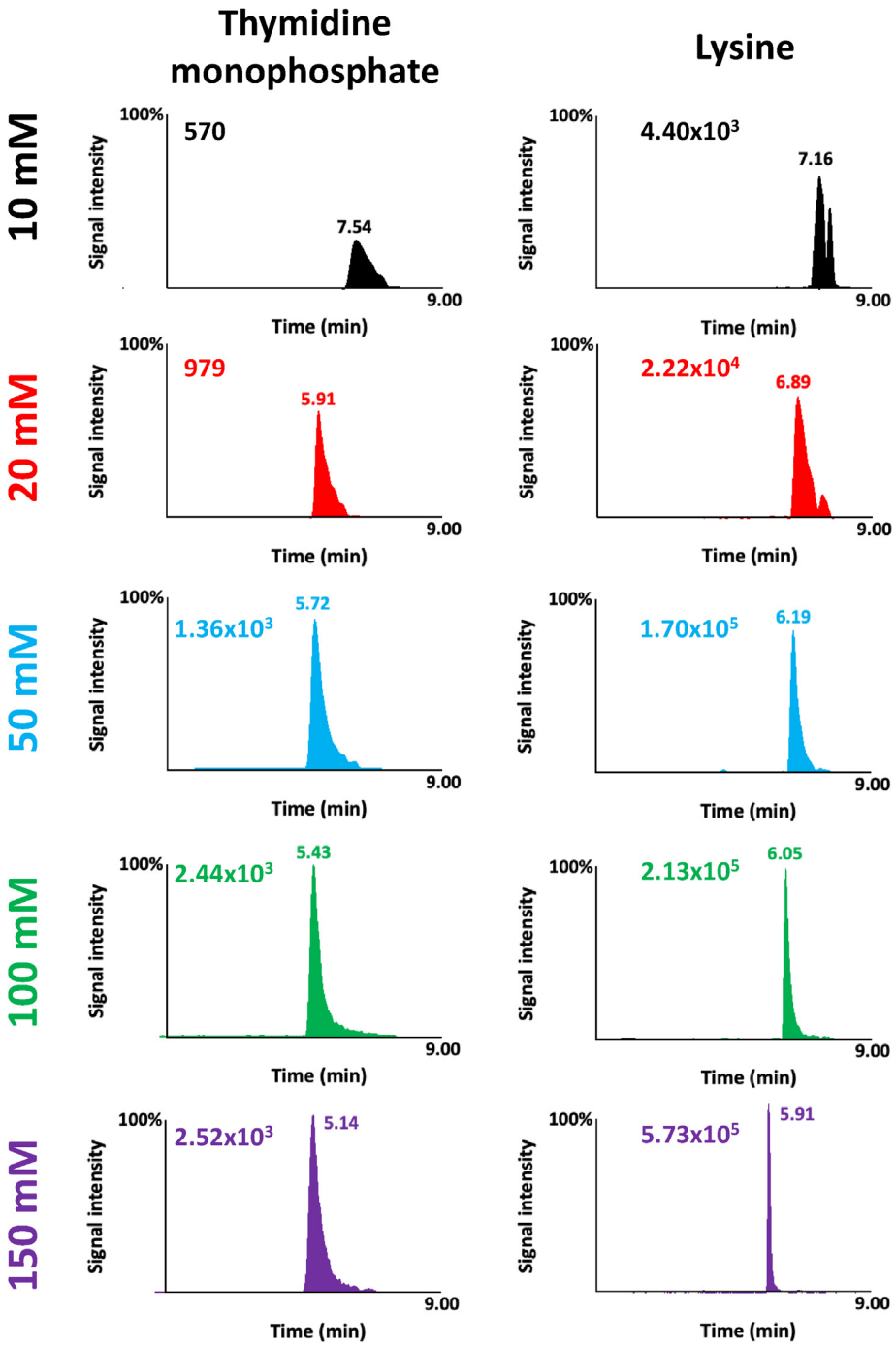

Fig. 3. Chromatograms for thymidine monophosphate (left) and lysine (right) obtained on a Poroshell HILIC column using concentrations up to 150 mM of ammonium formate in the mobile phase. Adapted, with permission, from Ref. [23].

higher column backpressure must be expected [23]. This is particularly relevant for columns packed with sub-2 μm fully porous silica particles having a low permeability [23,54]. Pressure values of 400 bar are quickly reached even at relatively low linear velocities. Unfortunately, the latest generation of UHPSFC equipment is only capable to handle column backpressures up to 400–660 bar. Therefore, if UC or EFLC-type gradients are considered, low flow-rates should be used to avoid system overpressure [23], as well as columns of reduced length [55]. Furthermore, it is also important to consider its impact on the kinetic performance. Some authors have recently attempted to understand how the kinetic performance of a column packed with sub-2 μm particles evolved during a UC-type gradient at four levels of co-solvent, *via* the analysis of van Deemter plots (Fig. 4) [54]. Under supercritical conditions (<5–10% of modifier), UHPSFC was able to provide low plate height values (H) at high velocities, meaning that high flow-rates must be employed to obtain the best performance. This statement is valid in particular for columns packed with sub-2 μm

fully porous silica particles. However, when transitioning progressively from a supercritical to a liquid-like state, the C-term region of the van Deemter curve rapidly increases, resulting in a worsening of the kinetic performance at high flow-rates [54]. This study indicates that, for UC-type gradients, the optimal velocity changes within the course of the analysis. Indeed, relatively low flow-rates are recommended at high co-solvent percentages, while high flow-rates can be used at low organic modifier percentage [23,54].

Unfortunately, as commercial UHPSFC systems do not possess an upper pressure limit as high as to those reached with UHPLC instruments (1200–1500 bar), alternative solutions have to be found to limit pressure and flow-rates issues. One of the most widely used solutions was to employ larger silica particles (3 and 5 μm), as observed in several articles [45,56–58], Another attractive solution is to select stationary phases based on superficially porous silica particles (also known as core-shell or fused-core). Their use is not widespread in UHPSFC, since there is only a limited number of articles using these type of particles, focusing either on chiral [34,36,59,60] or achiral applications [22,23,61]. Nevertheless, they can provide interesting advantages, especially when performing EFLC-type or UC-type gradients. The reduced backpressure, coupled to the possibility of obtaining comparable performance to those generated by fully porous sub-2 μm particles, should make superficially porous particles interesting in the context of UHPSFC [23]. Unfortunately, the commercial offer in terms of polar stationary phases packed with superficially porous particles is still too limited, and alternative strategies must be considered. Recently, Losacco et al. investigated the use of very high temperatures (up to 80 °C) in UHPSFC using a sub-2 μm stationary phase, focusing on the kinetic performance and pressure ranges available using modifier percentages as high as 100% [54]. Their results suggest that, with cosolvent amounts starting from 40 to 50% onwards, high temperatures (>40 °C) helped in maintaining good kinetic performance at high velocities (Fig. 4), as well as in reducing the backpressures generated when the mobile phase was predominantly composed of organic modifier. Besides the impact of column pressure drop, variations in the column temperature can also be helpful in optimizing the achievable selectivity. Finally, the stability of the stationary phase tested at 80°C gave very promising results.

3.2. Choice of UHPSFC stationary phases chemistry

The choice of the stationary phase chemistry in UHPSFC is not as straightforward as in UHPLC, where C_{18} selector is commonly used. Indeed, several UHPSFC column chemistries are available on the market, making the column screening protocol still very important [62]. Nonetheless, it is possible to summarize some common characteristics, to better understand the performance of UHPSFC for highly polar compounds. After its introduction and early development, SFC became an alternative to NPLC, in particular for chiral separation [8]. Hence, a significant part of the columns developed for UHPSFC present a relatively high polarity range with strong H-bonding properties, represented nowadays by bare silica based columns or those functionalized with various selectors such as diol or alkyl-pyridines [19,30,63]. All these stationary phase chemistries have been successfully used to efficiently analyze low to mildly polar compounds, but they should also work with more polar analytes, similarly to what is commonly seen with HILIC applications [19,22]. As previously discussed, the evolution of UHPSFC mobile phases with a more systematic use of water as well as high concentrations of additives made possible to demonstrate how UHPSFC stationary phases performed well also for more hydrophilic compounds. Among them, the most employed ones seem to be those possessing basic selectors (i.e. picolylamine, alkyl-pyridine and alkyl-amine), as they can provide an additional ionic interaction under acidic mobile phase conditions [32,57,64]. Nonetheless, those with a relatively neutral nature (i.e. bare silica, diol) are also widely used by research groups for the analysis of highly polar compounds [28,44,65]. In conclusion, while basic and/or neutral columns can be successfully employed for the analysis of highly polar substances, there is still the need to perform a screening procedure for a wide range of stationary phases to select the most appropriate one for a specific application.

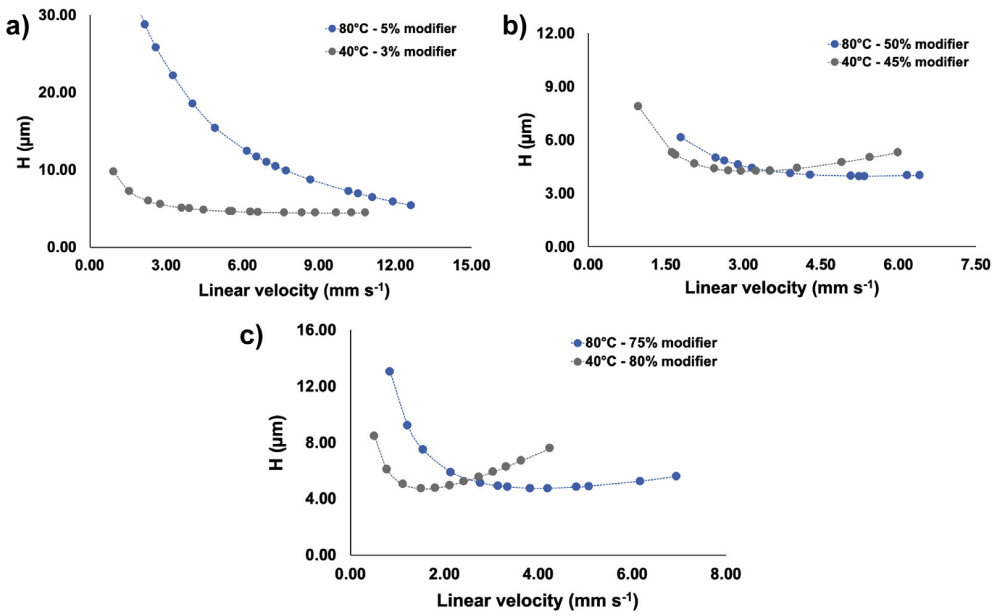

Fig. 4. Van Deemter plots obtained for a) butylparaben, b) maleic acid and c) indoxyl sulphate on the Torus 2-PIC 1.7 μm fully porous silica particles, using either 40°C (grey plots) or 80°C (blue plots) as the column temperature. Modified with permission from Ref. [54].

On the other hand, an increasing number of articles show promising results using alternatives column chemistries. As an example, due to their strong similarity, some researchers have highlighted the potential of HILIC columns under UHPSFC conditions [42,49,53,56,66]. Furthermore, UHPSFC allows an unprecedented flexibility in terms of column choice, compared to UHPLC. As an example, apolar stationary phases such as non endcapped C_{18} coupled to polar stationary phases, have been used for the analysis of saccharides [27]. Weakly apolar columns, such as pentafluorophenyl (PFP) and crown ether-based stationary phases were also used for polar metabolites and peptides analysis [21,31]. Last but not least, columns offering ion-exchange type retention mechanisms were also investigated and successfully used in UHPSFC for polar analytes [26,58].

4. Current applications and future trends

The evolution of UHPSFC in combination with modern stationary phases contributed to expanding the range of applications towards new areas. Previously considered as an analytical tool for apolar and mildly polar compounds, UHPSFC is now increasingly employed for analyzing polar analytes. UHPSFC has been successfully tested in the field of anti-doping analyses for either polar doping agents or related metabolites, in neat solutions or in complex matrices such as urine [20,25,26]. Its routine application has also been verified, demonstrating its complementarity to the more established UHPLC techniques [24,25]. UHPSFC has been also used to analyze amino acids [30,42,45,46] and vitamins in dietary supplements, as well as polar substances present in plant extracts [15,44,64,64,67] and food dyes [68].

More recently, UHPSFC has been more consistently used for the analysis of biomolecules such as nucleosides, peptides and, in few cases, even small proteins (Fig. 5) [22,23,31,32,69,70]. All these applications had already been tested in the past, however with the recent innovations in terms of analytical conditions, a renewed interest emerged. UHPSFC demonstrates interesting performance, leading up, potentially, to its more systematic use for such analytes. More information on the use of UHPSFC for the analysis of biomolecules is available in a recent review [13]. Another important field which has been recently explored in UHPSFC as an alternative analytical tool is metabolomics [19,65,71]. Here, UHPSFC provided good results, and enabled the simultaneous analysis of lipophilic and hydrophilic metabolites, demonstrating a good retention profile for a large library of more than 600 metabolites [22,23]. Finally, UHPSFC also demonstrated its value for the analysis of polar pesticides [72–75].

To further improve the resolving power in the field of UHPLC, a growing number of laboratories have started utilizing a multidimensional approach, by coupling complementary techniques to maximize the amount of information that can be obtained from a single run. Multidimensional chromatography involves, nowadays, the combination of two UHPLC modes such as, for example, RPLC, HILIC or ion-exchange [28,76]. The evolution of UHPSFC has definitely increased its attractiveness as one of the chromatographic dimensions in a multidimensional setup, coupled with a UHPLC technique or, in more extreme cases, to a second UHPSFC dimension [77–79]. Various technical impediments have slowed down the implementation of UHPSFC in multidimensional systems. However, recently there has been a stronger focus on the possibility of coupling UHPLC to UHPSFC in two-dimensional set-ups. Off-line hyphenation of UHPSFC to UHPLC has been, in a first instance, considered, as it presents less technical challenges [80,81]. Subsequently, on-line multidimensional systems with UHPSFC being either in the first or the second dimension have been studied [82–84]. Today, the most convincing application involving UHPSFC

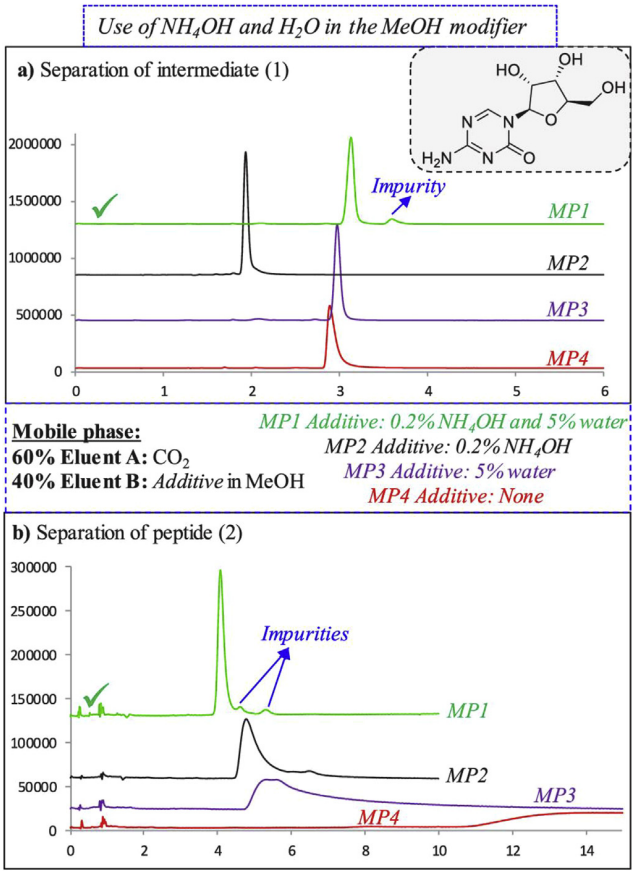

Fig. 5. UHPSFC analysis of a) nucleoside and b) peptide from related impurities using a water-rich modifier. Reprinted, with permission, from Ref. [70].

in one of the on-line dimensions consists in using achiral analysis in the first dimension and chiral separation in the second one, since it can benefit from the well-known advantages that UHPSFC provided in the context of chiral analysis. A more detailed review on this topic can be found elsewhere [77].

5. Conclusion

UHPSFC has witnessed an important transformation to improve its compatibility with strongly polar substances. Most of the work focused in developing alternative mobile phase conditions to boost the elution power in UHPSFC. The systematic addition of water, although in limited quantity, as well as the use of relatively high concentration of additives (>20–30 mM) have greatly contributed to enhance the ability of the $scCO_2$-based mobile phase in successfully eluting highly polar compounds.

Research groups focalized their attention also on the selected gradient profile. Two strategies have consequently emerged. While with EFLC-based gradients, the presence of CO_2 is strongly limited to allow a much higher percentage of water in the modifier, UC-based gradients aim to cover and analyze the widest possible range of molecules, from apolar to highly polar compounds, exploiting the ability of UHPSFC stationary phases to retain an incredible number of analytes, through H-bonds, dipole-dipole and ionic interactions.

As both EFLC-based and UC-based gradients need high percentages of liquid co-solvent, the backpressure values generated by UHPSFC columns, especially those packed with sub-2 μm fully

porous silica particles, are very high and therefore, alternatives have been evaluated. The solutions that have emerged focalize on either the employment of sub-3μm superficially porous silica particles or the use of high temperatures if sub-2 μm particles are chosen. With both approaches, good kinetic performance is maintained and it was possible, at the same time, to greatly reduce the column backpressure.

Finally, various applications involving UHPSFC for highly polar compounds have been developed. Thanks to the latest developments, UHPSFC demonstrated excellent results for different analytes, varying from amino acids, sugars, doping agents, pesticides, to biomolecules. Furthermore, the increased applicability of UHPSFC in different areas can fuel the interest of researchers in using UHPSFC in multidimensional systems, aiming in obtaining an analytical tool capable to generate an increased amount of complementary information within a single run.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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I.3 Coupling of UHPSFC to MS

Several detectors can be hyphenated to SFC, such as UV or ELSD. However, MS has recently captured the interest of the majority of research groups. Due to its unparalleled applicability range, as well as high selectivity and elevated sensitivity, MS represents the gold standard detector in numerous laboratories (28,29). Its coupling to chromatographic technique such as GC or, more importantly, LC has been the subject of different studies. Nowadays LC-MS represent the gold standard in metabolomics, anti-doping laboratories, food industry and for environmental analyses (28,30,31). Because of its usefulness, its hyphenation to SFC has been extensively tested. Due to the peculiar nature of the SFC mobile phase, specific interfaces have been developed throughout the years to increase the compatibility of SFC to MS, with several designs available on the market.

This section focuses on the current situation regarding the coupling of SFC to MS. Different MS ionization sources, as well as MS analyzers available on the market are described in the first half of this paragraph. A review article, providing a critical evaluation of SFC-MS and of its applications is presented at the end of this section.

I.3.1 MS ionization sources

Many typologies of MS ionization sources are available nowadays. Ionization chambers can work either under vacuum conditions, such as in the case of chemical ionization (CI) or electron impact (EI), or under atmospheric pressure (atmospheric pressure ionization, or API). The development and subsequent use of API sources, such as electrospray (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photo-ionization (APPI), allowed the coupling of separation techniques such as LC and, lately, SFC to MS systems. More specifically, ESI sources have proven to be decisive as they ensure the ionization and desolvation of a large spectrum of compounds, from small to large molecules, with intermediate to relatively low polarities.

ESI can be divided into three main phases (Figure 6). Initially, the mobile phase stream coming from the chromatographic system needs to be reduced into droplets released from the Taylor cone at the end of the capillary, with the aid of a nebulization gas as well as the use of high temperatures. These droplets will acquire a charge that can be either positive or negative, according to the applied capillary voltage. Subsequently, the charged droplets will undergo a reduction in their size, which would increase exponentially the ionic repulsion phenomenon as an excess of charges with the same polarity will accumulate on the surface of the droplets. This leads to Coulomb fission of the larger droplets into smaller ones, igniting a chain of reactions which would lead, eventually, to the creation of gas-phase ions (32). Finally, the ions

in gas-phase will enter into the MS analyzer (under vacuum region), leading to their separation and detection.

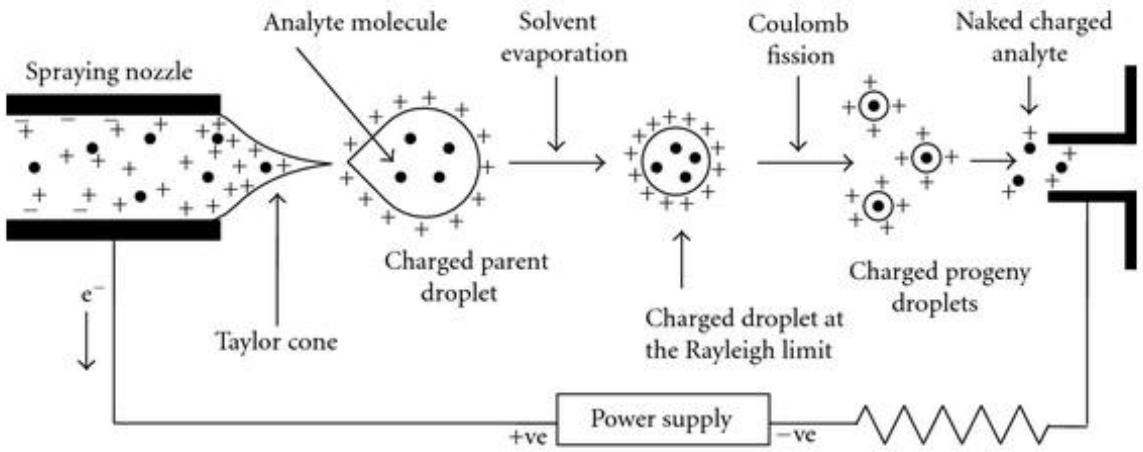

Figure 6: Representation of the ESI process. Reprinted from (33)

While the working principle of ESI source does not change between LC and SFC, some considerations need to be made on the state of the mobile phase reaching the ionization chamber. In LC, the solvent is always in a liquid state, thus the ESI source needs to be highly efficient in generating the charged droplets and in reducing their size, to have consequently the production of gas-phase ions. Therefore, an important parameter in LC-MS analysis is the flow-rate employed during the analysis. Flow rates above 500-700 µL/min tend to be considered detrimental for LC-MS, as the ionization process would not be able to efficiently generate the charged droplets from the solvent entering into the ESI capillary. This leads to a general reduction in the MS sensitivity. Consequently, it is advised to work below such flow-rates (e.g. 250 µL/min) in LC-MS. With SFC, on the other hand, the situation is more complex. As discussed in the previous section, this technique needs to work at high velocities (thus higher flow rates) to obtain the maximum possible chromatographic efficiency. Therefore, it seems at first that SFC does not fit well with ESI sources. However, the different SFC-MS interfaces currently available on the market tend to have a flow splitter before delivering the solvent in the ionization chamber (34,35). More details on the different SFC-MS interfaces available are given in the review article in section I.2.4. In particular, the "*pre-BPR splitter with sheath pump*" geometry used by Waters and Agilent on their SFC systems limit the flow-rates of modifier entering the ESI chamber to less than 250 µL/min, using various co-solvent percentages up to 40% in the mobile phase (Figure 7).

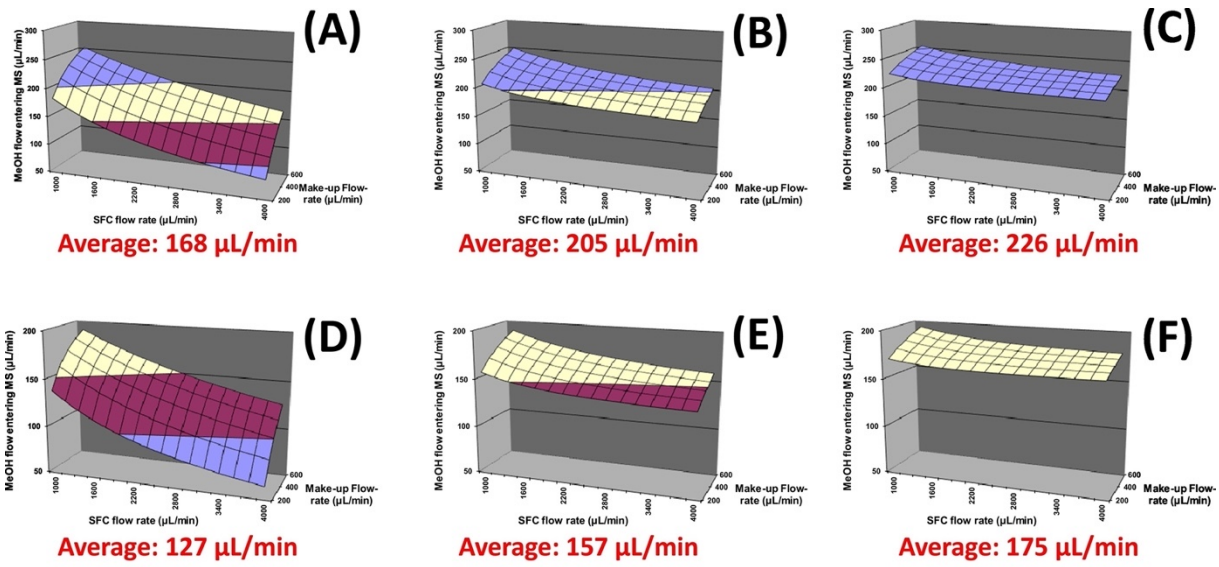

Figure 7: Simulated amount of MeOH entering into the MS as a function of the flow rates on the SFC pump and the sheath pump, with the Waters "pre-BPR splitter with sheath pump" interface (A–C) and Agilent "pre-BPR splitter with sheath pump" interface (D–F). Simulations have been performed with a mobile phase composed entirely of $scCO_2$ (left column), 80% $scCO_2$ and 20% MeOH (middle column), 60% $scCO_2$ and 40% MeOH (right column). Adapted, with permission, from (35).

A second factor also contributes to the lower amount of solvent reaching the ionization chamber compared to LC-MS. In the tubing employed to connect the SFC-MS interface to the ESI source, carbon dioxide experiences a state shift from a supercritical fluid to a gas, as it is not anymore under the influence of the BPR module. Therefore, when the mobile phase reaches the capillary, the CO_2 is immediately removed and the actual amount of solvent entering into the ESI source is only composed of MeOH and eventual additives. Furthermore, the gaseous CO_2 can improve the efficiency of the ionization chamber in the evaporation of the liquid solvent, with the potential to lower even further the limits of detection. More details on the sensibility achievable with SFC-MS compared to LC-MS are given in the review article of paragraph I.3.4.

I.*3.2 MS analyzers*

Once ions are passed in their gaseous state and have acquired a charge, they will be transferred in the mass analyzer of the MS instrument. Mass analyzers are fundamental, since their role is to guarantee the discrimination between the different ions that have been previously generated in the MS ionization source. Mass analyzers are classified according to their resolution power, following equation 3:

$$resolution\ power = \frac{m}{\Delta m} \quad (3)$$

where m is the ion's mass and Δm is the resolution or full width at half maximum (FWHM). Using equation 2, it has been possible to classify mass analyzers in two main groups: those with a resolution power < 10 000, called low-resolution mass analyzers, and those with a resolution power > 10 000 (high-resolution mass analyzers). In the first group quadrupole (Q), and ion trap (IT) analyzers are present. These are not capable to discriminate ions with the same nominal mass, due to the unit resolution they offer. In compensation, they are able to provide excellent performance in the context of targeted analyses, especially when quantification is needed. High resolution mass analyzers, such as time-of-flight (TOF) or Orbitrap©, are capable in separating ions down to 0.001 atomic mass unit of difference and are, therefore, mainly used in untargeted analyses to obtain information regarding the structure of unknown analytes. In this thesis work, single quadrupole, triple quadrupole and time-of-flight analyzers have been employed. More details regarding how they function and their application with chromatographic systems such as LC and SFC can be found in the previous thesis works of Dr. Alexandre Grand-Guillaume Perrenoud (University of Geneva, Thesis n°4717) and Dr. Vincent Desfontaine (University of Geneva, Thesis n°5181), as well as in the review article present in paragraph I.3.4.

I.3.3 Matrix effect

Application involving SFC-MS have started to greatly increase with the development of the latest generation of SFC-MS interfaces. Among those, the analysis of biological matrices has attracted the attention of researchers. Indeed, the possibility to benefit from the complementarity given by SFC in the chromatographic separation, in combination with the typical detection range of MS was seen by many as an interesting alternative to LC-MS. However, when compounds present in complex matrices are analyzed with MS, a phenomenon called matrix effect (ME) starts to arise. Matrix effect has been defined as the alteration of the ionization process due to the presence of co-eluting substances generally invisible in chromatograms (36). ME has been known for several years in LC-MS, with the first example described by Tang and Kebarle in 1993 (37). Two ME types have been identified so far: ion suppression, in which the signal intensity of the desired ion present in the matrix is lower than the signal generated by the same analyte as a standard, both at identical concentration levels. The second ME phenomenon is ion enhancement, in which an increase of the signal intensities can be observed, in opposition to ion suppression. Although almost 30 years have passed, there is no clear theory that can fully explain how ME actually occurs. The main hypothesis states that ME is due to a competition phenomenon between non-volatile matrix components and the ions of interest at the droplet surface for their subsequent transfer in the gaseous state and, thus, entrance in the MS analyzer. However, while this hypothesis

can be useful in understanding ion suppression, it does not correlate with the ion enhancement phenomenon.

Several strategies have been developed to provide an estimation of the ME generated during the analysis. One of the most common approach was introduced by Matuszewski *et al*. (38), in which the ratio between the peak area of one analyte spiked *after* the extraction procedure applied on the biological matrix over the peak area of the same analyte, at the same concentration, in neat solution is calculated. ME is, then, obtained using the following equation 4:

$$ME\ (\%) = \frac{Peak\ area\ of\ post\ extraction\ spiked\ sample}{Peak\ area\ of\ standard\ in\ neat\ solution} * 100 \quad (4)$$

Another methodology was introduced by Bonfiglio *et al* (39), in which a post-column infusion of the analyte is made during injections of the biological matrix and neat solvent (Figure 8).

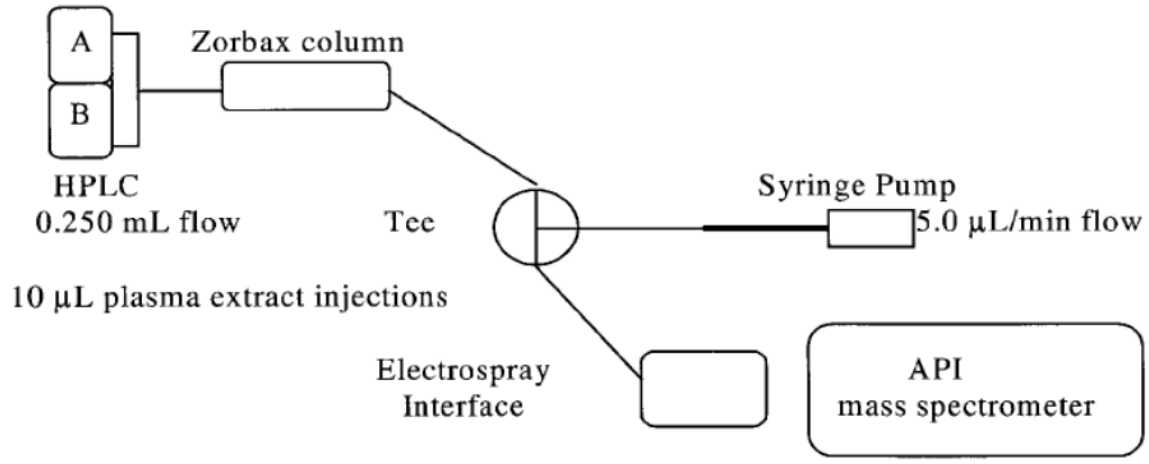

Figure 8: Post-column infusion system. Reprinted from (39).

As previously mentioned, ME is due to the coelution phenomenon of the desired analyte with substances present in the tested biological matrix. As the latter can interact with the stationary phase, it is expected that the choice of the chromatographic technique can impact the level of ME generated within the analysis, thus reducing its extent. SFC, therefore, can provide a quite different ME profile compared to that of RPLC and HILIC, due to its orthogonality and separation complementarity. Additional information are present in the review article of paragraph I.3.4.

I.3.4 Review article: Recent trends for SFC-MS

In the following review article, published in 2019, different aspects related to SFC-MS analyses, such as the type of SFC-MS interface, matrix effect as well as achievable sensitivity compared to LC-MS? are discussed. Examples of the latest applications using SFC-MS are also given.

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Supercritical fluid chromatography – Mass spectrometry: Recent evolution and current trends

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ABSTRACT

Supercritical fluid chromatography (SFC) has recently experienced renovated impulse from research groups. Its hyphenation to mass spectrometers (MS) proved to be of significant importance in catalysing interest from researchers. In contrast to liquid chromatography (LC), the coupling of SFC-MS requires the use of an interface in order to deal efficiently with the decompression of supercritical CO_2 and possible precipitation issues of samples while entering the ionization chamber. The most common SFC-MS interfaces employ an additional sheath pump that reduces sample precipitation. However, there are still issues in dealing with the CO_2 decompression phenomenon, with different solutions being given. Matrix effects (MEs) under SFC-MS have proved to be quite different from those generally observed in LC-MS, with ion suppression being the main form of ME. Nonetheless, SFC-MS is capable of reaching comparable sensitivity values to LC-MS, and in some cases performing even better. Several applications have been recently developed for SFC-MS, spacing from the analysis of plant extracts, biological matrices for anti-doping and forensic purposes, as well as highly polar compounds such as carbohydrates and endogenous metabolites.

1. Introduction

The use of mass spectrometers (MS) as a detector hyphenated to chromatographic separation has known an incredible growth, in the recent years, thanks to its high versatility, sensitivity, and range of possible applications [1,2]. Considering the diversity of MS analyzers present on the market (i.e. single and triple quadrupole, time of flight, ion trap, Orbitrap and hybrid instruments), it is possible to perform qualitative and quantitative analysis at very high sensitivity [3–5], as well as to generate elevated MS resolution between compounds having very similar mass-to-charge values [6,7].

One of the most successful marriages between chromatography and mass spectrometry is now represented by the hyphenation of liquid chromatography (LC) with MS [8–10]. This coupling became possible thanks to the development of atmospheric pressure ionization (API) sources such as electrospray ionization (ESI) [11,12] and atmospheric pressure chemical ionization (APCI) [13]. Besides LC, other separation techniques have also been successfully hyphenated to MS, including gas chromatography (GC) [14] capillary electrophoresis (CE) [15], and supercritical fluid chromatography (SFC) [16,17]. SFC was initially developed during the 1960s and regained the attention of several research groups starting from the 1980s [18], but the interest remained limited to chiral separation [19] and preparative chromatography [20], due to a lack of robustness and sensitivity of the instrumentation [21]. Since 2012, a new generation of SFC instruments was introduced on the market. These new systems possess various desirable features, such as i) reduced system volume and a relatively high upper-pressure limit, compatible with columns packed with sub-2 μm particles, ii) improved robustness and iii) easy MS hyphenation [22]. This allows SFC to transition into ultra-high performance supercritical fluid chromatography (UHPSFC) [18], in a similar way to what has been witnessed with LC since 2004, with a consequent increase in terms of interest and publications being made. A quick analysis made on the main research platforms currently available shows a gradual but constant increase in articles which have keywords such as "supercritical fluid chromatography" or "SFC", in the period from 2012 (343 publications) till 2018 (634 publications). This chromatographic technique has the peculiarity of employing a supercritical (or often subcritical) mobile phase, thanks to the use of carbon dioxide in its supercritical state as major constituent [18,23]. In modern UHPSFC, carbon dioxide is always mixed with an organic

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modifier, usually methanol, which ensures the complete elution of compounds from low to high polarity [23]. Some salts (i.e., ammonium formate, ammonium hydroxide …), as well as acids (i.e. formic acid, trifluoroacetic acid …) or bases (i.e. ammonium hydroxide, diethyl- and triethylamine …), and a small amount of water could also be added to the mobile phase to improve method repeatability and peak shapes of ionizable substances. Finally, the use of a supercritical mobile phase presents several advantages in chromatography, including a minor environmental impact compared to organic solvents such as *n*-hexane or *n*-heptane, low viscosity, high diffusion coefficients, and high density, thus enabling SFC to combine the advantages of LC and GC [18,23].

The aim of this review was to describe the latest developments related to the hyphenation of UHPSFC and MS, highlighting some advantages that this technique can offer in contrast with the current state-of-the-art techniques. First, a detailed description of the UHPSFC-MS interfaces available on the market will be provided, including some potential issues related to the use of a supercritical fluid. Secondly, the influence of the make-up solvent nature and the evaluation of matrix effects will be assessed. Then, a comparison of achievable sensitivity in UHPSFC-MS and UHPLC-MS will be performed. Finally, an overview of some relevant applications that have been developed in the last few years will also be given.

2. UHPSFC-MS interfaces

The hyphenation of UHPSFC with MS is not as straightforward as with LC/UHPLC instruments. Indeed, supercritical fluids possess much higher compressibility than liquids, that needs to be controlled, particularly when the fluid is not anymore under the backpressure control [24]. Indeed, when the pressure is released, analytes can precipitate before entering the MS instrument. Besides the regulation of backpressure, the interface should also help to improve the ionization yield in ESI, particularly when the mobile phase is composed of a high proportion of CO_2. Lastly, the chromatographic integrity (retention, selectivity, and efficiency) should also be maintained when MS detection is used. For all these reasons, the providers of SFC instruments have developed several interface schemes over the years (Fig. 1), able to solve these different issues [24,25].

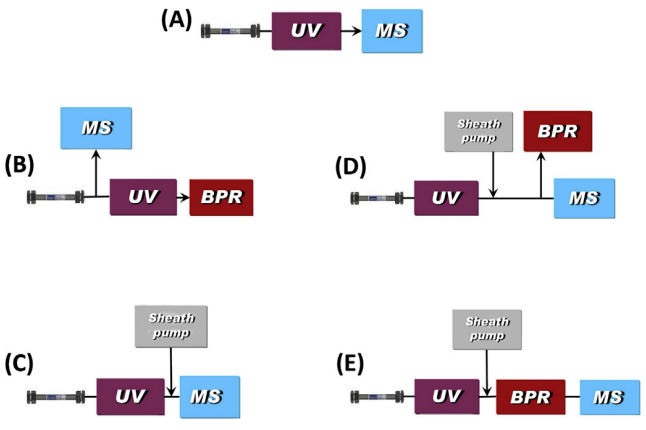

Fig. 1. Representations of the five most common SFC–MS interfaces. (A) *"direct coupling"* interface, (B) *"pre-UV and BPR splitter without sheath pump"* interface, (C) *"pressure control fluid"* interface, (D) *"pre-BPR splitter with sheath pump"* interface, (E) *"BPR and sheath pump with no splitter"* interface. Reprinted from *J. Chromatogr. B*, Vol. *1083*; D. Guillarme, V. Desfontaine, S. Heinisch, J.-L. Veuthey; What are the current solutions for interfacing supercritical fluid chromatography and mass spectrometry?, pp 160–170 [ref 25]. Copyright 2018, with permission from Elsevier.

The most common interface available on the market is known as the "*pre-BPR splitter with sheath pump*", commercialized by Waters and Agilent [25] (see Fig. 1D). This interface consists of two zero-dead-volume (ZDV) T-unions linked in series, allowing the addition of a make-up solvent from a sheath pump (first ZDV T-union) and the use of an active-backpressure regulator (ABPR) (second ZDV T-union), to direct only a limited part of the flow-rate into the MS ionization source, while the remaining part goes to the waste. This interface offers the obvious advantage of reducing the possible precipitation of samples in the mobile phase. In addition, thanks to the flexible BPR regulation and the presence of the sheath pump, it also allows sending a highly suitable mobile phase flow rate and composition to the ESI sources, thus producing an excellent sensitivity [25].

The second available interface, among the most popular ones, is called *"BPR and sheath pump with no splitter"*, commercialized by Shimadzu and Agilent (see Fig. 1E). In this configuration, there is only one ZDV T-union, used to deliver the make-up solvent. This interface, which does not possess flow-splitting, is well suited for APCI-MS, which is a mass flow dependent device, since it delivers the entire sample to the MS [24]. Moreover, the last tubing entering into the ionization source passes through the BPR, which is heated at a relatively high temperature (around 50°C), to limit decompression cooling phenomenon and solute precipitation. Until now, this interface has been rarely employed for real applications, and therefore its advantages and drawbacks are still not well identified [25].

A remark has to be done on the Agilent SFC-MS interface, since it is the only one that allows the user to choose between the "*pre-BPR splitter with sheath pump*" and the *"BPR and sheath pump with no splitter"* configurations.

Besides these two interfaces, there are also a few other solutions that have been described for hyphenating UHPSFC and MS, but they present some major issues, making them inferior to the ones previously described. More details on the different interfaces currently available can be found in a recently published review from our group [25].

When hyphenating UHPSFC and MS, several important issues need to be considered [24–26]. In the two previously described interfaces, the BPR module is located before the MS. Therefore, there is no control over the mobile phase state entering the ionization chamber. In this part of the setup, the CO_2 is not under the influence of the BPR and should decompress endothermically, which leads up to different problems [24]. First, the decompression, followed by a drop in the temperature at the connector level, increases the risk of analyte precipitation [24,25]. Moreover, the addition of the make-up solvent, necessary to replace decompressed CO_2, might be insufficient to ensure the solubility of the samples, leading to possible precipitation issues [27]. Another issue related to the uncontrolled CO_2 decompression is the possible peak broadening that has been previously reported [24]. This phenomenon could be attributed to different factors: the temperature drop is certainly one of them, since it increases solvent viscosity and thus reduces analytes diffusion coefficient [24]. In addition, considering that there is no pressure and temperature control in the tubing located after the BPR, phase separation is most likely to occur between the liquid organic modifier and gaseous CO_2 [24,25,27]. As described elsewhere [24], to better understand the influence of the phenomena described above, it is advised to follow the vapor-liquid equilibrium (VLE) curves for CO_2+methanol mixtures. Different situations can be foreseen: the flow patterns can greatly change, with the formation of CO_2 bubbles of different diameters based on the volume ratios between the gas and the liquid. This, consequently, affects the linear velocity of the flow entering the MS. Linear velocity is also influenced by the change in surface tension, viscosity and other parameters which are not behaving as expected

[24]. The phase separation can be another potential problem that should not be underestimated, especially since it might lead to more severe issues such as band broadening or even loss of the chromatographic separation.

Different solutions have been found to tackle these drawbacks observed in the SFC-MS setup. For the precipitation issue, the addition of a sheath pump, which continuously delivers a make-up solvent (i.e., methanol, methanol + buffer, methanol + small amount of water), was found to be a good solution [27]. Indeed, the delivery of a methanol-rich solvent strongly limits the precipitation of polar compounds, without sacrificing too much SFC and MS performance. The addition of a make-up solvent, however, may lead to the insurgence of another potential problem: a dilution factor can appear which could negatively affect MS sensitivity, especially on concentration-dependent ionization sources such as ESI [25,27–29]. Contrary to what could be expected, the dilution factor remains always reasonable, whatever the mobile phase and make-up conditions, thanks to the use of the active BPR [27,30]. Regarding the management of CO_2 decompression, a solution is to modify the interface [6,31]. One key parameter is obviously the temperature that needs to be controlled, to avoid phase separation. As described in more details elsewhere [24], heating is not always the best choice. The use of combined isenthalpic and isopycnic plots, for mixtures of CO_2/methanol with fixed compositions, clearly highlight that cooling, instead of heating, should be preferred [24]. Indeed, the analysis of these plots definitely indicates that, by lowering the temperature, it is possible to avoid the area in which phase separation occurs for a greater range of pressure values [24]. The temperature reduction, therefore, translates into a wider range of the CO_2 decompression. Density, also, does not change, which therefore translates in much fewer precipitation issues of several compounds, which were soluble with a high-density mobile phase. The other parameter is the interface geometry; indeed, changing the geometry of the capillaries used in the interface (i.e., length, inner diameter, etc.) can be an easy solution to maintain a constant mobile phase density. Only two papers, however [6,31], describe the evaluation of a new capillary restrictor for ESI interfaces, able to reduce the pressure drop in the connector. According to the authors, the new interface design has brought a more stable ESI spray, positively affecting the peak shapes and repeatability, thus allowing a better quantification of the compounds that have been tested [6,31].

3. Matrix effects in SFC-MS vs. LC-MS

UHPLC coupled to ESI-MS and tandem ESI-MS/MS instruments, is one of the most successful analytical techniques for the analysis of endogenous and exogenous compounds in complex matrices, such as urine, plasma or plants extract [32–34]. However, when analyzing biological matrices, it is important to consider the possible enhancement or suppression of analytes signals in the ionization stage by compounds that are present in the matrix, and co-elute with the investigated compounds [35,36]. This effect, better known as the matrix effect (ME), negatively affects the quantification of substances present in such matrices. Indeed, a signal suppression or enhancement of targeted substances has an obvious impact on LODs/LOQs and may increase variability on peak areas. Therefore, validation of the analytical method can become challenging. Since the retention mechanism in SFC on polar stationary phases (mostly polar interactions) is orthogonal to LC (mainly hydrophobic interactions), coelution of investigated compounds and substances contained within the matrix may be very different. Therefore, UHPSFC-ESI-MS(/MS) can be considered as a useful strategy to minimize or at least modify the impact of ME, in comparison with UHPLC-ESI-MS(/MS) [37].

In the last 3–4 years, there has been an increasing number of studies dealing with the application of UHPSFC-MS for the analysis of biological matrices [38–42]. Urine has been by far the most widely used matrix, due to its relative easiness of collection and sample treatment. In the case of urine, ME is mainly due to the presence of polar compounds such as urea, creatinine, glucuronic acid, uric acid, etc., as well as salts. Svan et al. [36] have recently made a systematic comparison of ME between RPLC-MS and SFC-MS, using 11 representative drugs in urine samples. In their study, ME was evaluated using the post-column infusion matrix profiles approach. To explain the differences observed in terms of ME, the authors first described the modification in separation profiles of matrix components between the two chromatographic techniques. Indeed, compounds generating ME in urine, which are highly polar, are eluted quite early in RPLC conditions, while they are strongly retained on SFC conditions and lately eluted thanks to the increasing concentration of the polar organic modifier in the mobile phase [36]. The differences, however, are not limited only to the separation profiles. In fact, under SFC conditions, there is a clear predominance of the ion suppression phenomenon, whose origin was further investigated in a follow-up paper [43]. In RPLC, both types of MEs (ion suppression and enhancement) co-exist, depending on the investigated analyte [36]. A second paper [37] correlated MEs obtained in RPLC and SFC using two different sample preparation methodologies (non-selective and selective), and the Matuszewski's approach was used as the ME evaluation. The conclusions reached by both authors were similar, clearly stating that signal suppression is the major type of ME in SFC for urine [37]. Moreover, SFC has proved to give less ME than RPLC in all experiments with urine samples [37]. This statement is further confirmed in other papers, where ME was found to be quite low in SFC-MS conditions [38,44,45].

While using plasma, however, the situation seems to be different. Indeed, the ME generated by plasma for around 40 representative drugs in SFC and RPLC [37] gave unexpected results. Higher signal suppression was observed in RPLC *vs.* SFC with the selective sample preparation methodology (solid phase extraction, SPE). However, the impact of ME was also highly dependent on the selected column chemistry in SFC [37]. In another study, the use of protein precipitation (PP) for plasma sample brought results that are similar to urine, with signal suppression being more common in SFC [36]. A third paper dealing with the application of SFC for the determination of three major antiepileptic drugs in plasma reports the level of ME around 95–100%, with only one compound subjected to slight signal suppression, stating therefore that SFC does not present issues with ME in plasma [46]. To draw some reliable conditions on ME for plasma samples, there is, however, a need for more experimental results and discussion, due to the limited number of applications reported with human plasma under SFC conditions. In addition, it is also important to keep in mind that ME may be highly dependent on the geometry of the electrospray ionization source.

4. Achievable sensitivity in SFC-MS vs. LC-MS

SFC has always been considered as a well-suited technique for MS detectors, thanks to the hybrid nature of the mobile phase, and the use of organic solvents (mostly methanol) with higher volatility than water, thus positively influencing the ionization process, especially in ESI mode. The recent introduction of modern and reliable UHPSFC-MS systems allowed to experimentally prove some of the potential benefits of SFC over LC. Indeed, as shown in Refs. [28,47–50], excellent values for LODs and LOQs were met, with LOD values often down to below 1 ppb [28]. However, SFC-MS does not systematically provide a clear advantage over LC-MS in terms of sensitivity. Indeed, it was found that, while with the older

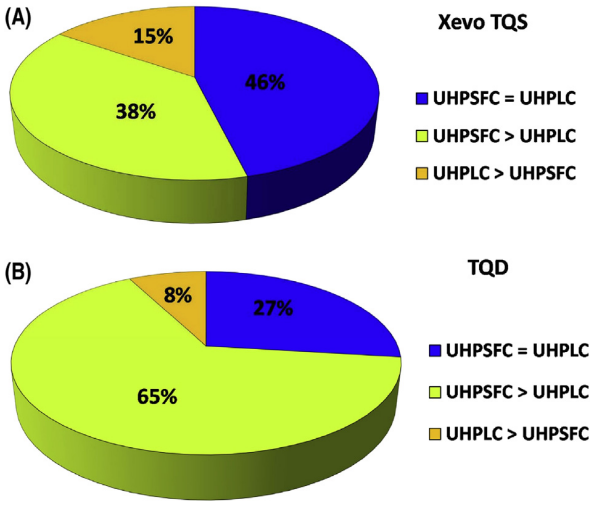

Fig. 2. A comparison of sensitivity between two different triple quadrupole platforms, i.e., Modern MS/MS device, namely Waters Xevo TQ-S (A) and old-generation MS/MS device, namely Waters TQD (B) in UHPSFC–MS/MS and UHPLC–MS/MS modes. Data used for this comparison were taken from Ref. [61]. Reprinted from *Anal. Chim. Acta*, Vol. *853*; L. Nováková, M. Rentsch, A. Grand-Guillaume Perrenoud, R. Nicoli, M. Saugy, J.L. Veuthey, D. Guillarme; Ultra high perfomance supercritical fluid chromatography coupled with tandem mass spectrometry for screening of doping agents II: Analysis of biological samples; pp 647–659 [ref 50]. Copyright 2015, with permission from Elsevier.

generation of MS instruments, SFC generally provides a higher sensitivity than LC, with the more recent mass spectrometers, SFC and LC were found to give very close results (Fig. 2) [51]. This observation was explained by the use of improved ionization sources on the more recent MS instruments, making them more able to handle higher proportion of water [51]. As an example, it was found that, out of 43 anabolic agents tested in human urine, LC provided a sensitivity level equal to 0.1 ng/mL for 98% of the analyzed compounds, while in SFC this percentage was reduced to 76% [52]. A similar result was obtained for vitamin D metabolites, with worse LLOQs in SFC than LC [53]. The main reason for these negative results is related to the limited injection volume in SFC. Indeed, it is well known that a lower injection volume has to be used in SFC vs. LC, especially when using polar and polar protic solvents such as methanol or water as the injection solvents [52–54], which should obviously negatively affect sensitivity. Moreover, different column geometries are generally used in LC and SFC (2.1 mm and 3.0 mm as internal diameters, respectively), which could further increase the dilution factor in SFC and reduce achievable sensitivity [52].

As previously discussed, there is a need to use a make-up solvent to couple SFC with MS. This means that users have the possibility to modify the mobile phase composition before entering MS detection, so that the ionization process can be enhanced, especially in ESI mode. Some authors have recently demonstrated how the addition of either small quantities of water or the use of additives/buffers in the make-up pump, increased the MS signals, thus improving sensitivity [28,48]. Using a wide range of endogenous steroids, the authors screened different buffers/additives in the make-up solvent, finding that either pure ammonium fluoride or ammonium fluoride mixed with formic acid in the solvent, can greatly improve ionization efficiency in ESI mode for steroids. In another work, it has been highlighted how the make-up solvent can positively influence the ionization of protease inhibitors in ESI conditions, with a simple tuning of its composition [27]. The authors have concluded that, while in LC, the mobile phase composition is not easily modifiable to enhance MS performance, the necessary addition of the make-up solvent in SFC can generate large MS signals increases, also allowing the possibility of considering post-column derivatization to improve further MS detection [27].

5. Applications of SFC-MS

As already observed for SFC-UV, there has been a constant and impressive increase in the number of new applications recently developed in SFC-MS.

An important field of application is the analysis of natural products. Indeed, there have already been developments and successful implementations in the past, however, now the constantly growing use of high resolutions MS instruments (HRMS), hyphenated not only to LC but also to SFC, has pushed the latter even further in this area. Besides the analysis of lipophilic compounds including lipids in plants [55–58], there is an interesting and growing trend,

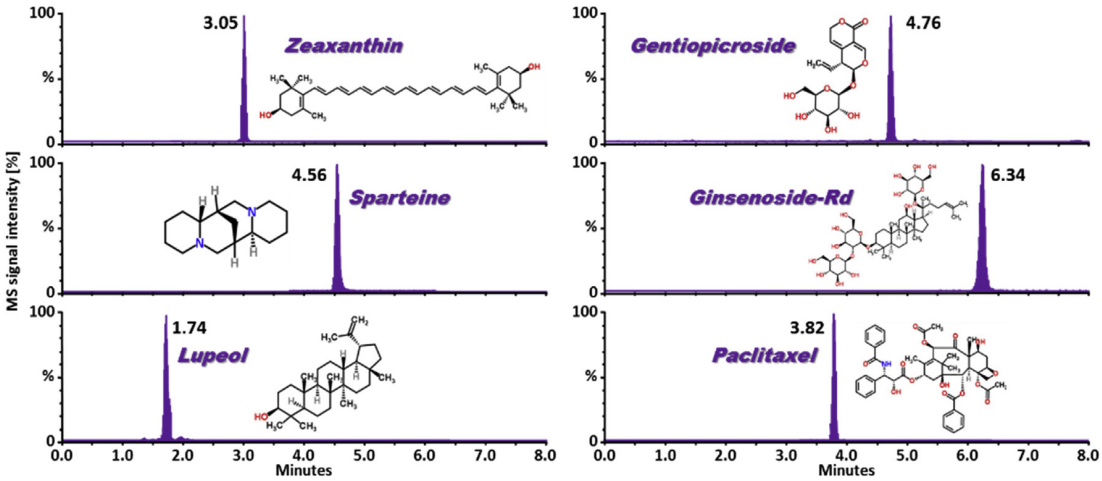

Fig. 3. Example of chromatograms (UHPSFC-QqToF-MS traces) obtained on Diol column, highlighting chemically diverse compounds in terms of Log P, H-bond capability and molecular mass respectively: carotenoid zeaxantin (10.92, 2, 568.43 Da); alkaloid sparteine (2.84, 2, 234.21 Da); triterpenoid lupeol (10.46, 2, 426.39 Da); the iridoid gentiopicroside (−3.03, 13, 356.11 Da); saponin ginsenoside-Rd (3.38, 30, 946.55 Da); diterpenoid paclitaxel (3.95, 19, 853.33 Da). Reprinted from *J. Chromatogr. A*, Vol. *1450*; A. Grand-Guillaume Perrenoud, D. Guillarme, J. Boccard, J.-L. Veuthey, D. Barron, S. Moco; Ultra-high performance supercritical fluid chromatography coupled with quadrupole-time-of-flight mass spectrometry as a performing tool for bioactive analysis; pp 101–111 [ref 60]. Copyright 2016, with permission from Elsevier.

namely the analysis of compounds with increasing polarity, such as monosaccharides [59], saponins [60] and flavonoids [61]. Other natural compounds are also being analyzed under SFC-MS, such as plant metabolites with interesting potential as drugs (Fig. 3) [62,63]. A specific category, which also attracts attention, is cannabinoids; indeed, the use of this class of compounds is rapidly increasing, in both medical and forensic applications [64–66]. Today, SFC-MS can be considered as a complementary technique to LC-MS, with an interesting ability in obtaining resolution of positional isomers and diastereomers, with a high degree of orthogonality to LC [67]. Moreover, the methods developed in SFC-MS also fit well with quality control requirements of real-life cannabis samples analysis [49], thanks to an easier sample preparation phase and a robust, fast and generic analytical method [49].

A second application area that is being under constant development is the implementation of SFC-MS in the forensic and anti-doping control analysis. Indeed, there has been an important number of papers recently released and focusing on several classes of compounds: amphetamines [45,68,69], stimulants and sympathomimetic drugs [51,70,71] or anabolic agents and steroids [44,52,72,73] (Fig. 4). Researchers involved in the field of anti-doping analysis are now testing new analytical techniques (such as SFC-MS), to find possible advantages to the current state of the art represented by LC-MS. Furthermore, SFC is not only being used as an analytical method but also employed in the sample preparation stage [73], with the aim to replace older methods employed in the sample treatment. Obviously, SFC-MS methods that wish to be employed in anti-doping laboratories also have to be validated. This aspect is being currently investigated by several authors, with a growing number of publications [38,39,45,47,49,74] showing that the validation procedure in SFC-MS yields similar, if not even better results than LC-MS. Indeed, during different validation processes of SFC-MS methods, it was found that SFC-MS manages to provide better results in terms of identification, reproducibility, precision and accuracy when compared to LC-MS [47,74]. These findings are extremely important in establishing SFC-MS itself as a technique that is compatible with regulated bioanalytical laboratories.

Another arising trend in SFC-MS applications is the analysis of hydrophilic and highly hydrophilic compounds under subcritical conditions [40,75]. SFC has been historically considered as a substitute technique to normal phase LC, and therefore, it has been mostly used for the analysis of compounds with low to medium polarity. However, thanks to the development of innovative strategies, such as the addition of small amount of water and/or salts in the organic co-solvent, as well as the use of gradient conditions up to 70–100% organic modifier, the range of analyzable molecules can be extended to molecules possessing log *P* values below 0 [75]. Thanks to this new possibility, SFC-MS is now shifting towards the analysis of compounds that classically fall under the domain of HILIC-MS. As example, SFC-MS is now increasingly employed in the field of metabolomics [75], in particular for the analysis of amino acids [40,76] and carbohydrates [75,77]. In addition, due to the high versatility of SFC-MS, it can be successfully employed for the simultaneous analysis of both hydrophilic and lipophilic molecules, from carbohydrates to lipids in metabolomics [75] (Fig. 5), from water to fat-soluble vitamins in food [78], and from highly hydrophilic to lipophilic trace organic compounds in environmental samples [79]. As more applications involving the use of SFC-MS with polar and highly polar compounds are arising, it can be stated that SFC-MS has now become a well-suited technique not only for lipophilic compounds, but also for those analytes whose polarity falls between $-2 < \log P > 2$. A recent review on the latest applications developed in SFC-MS for natural products, food and environmental analysis as well as bioanalysis and metabolomics is now available [80].

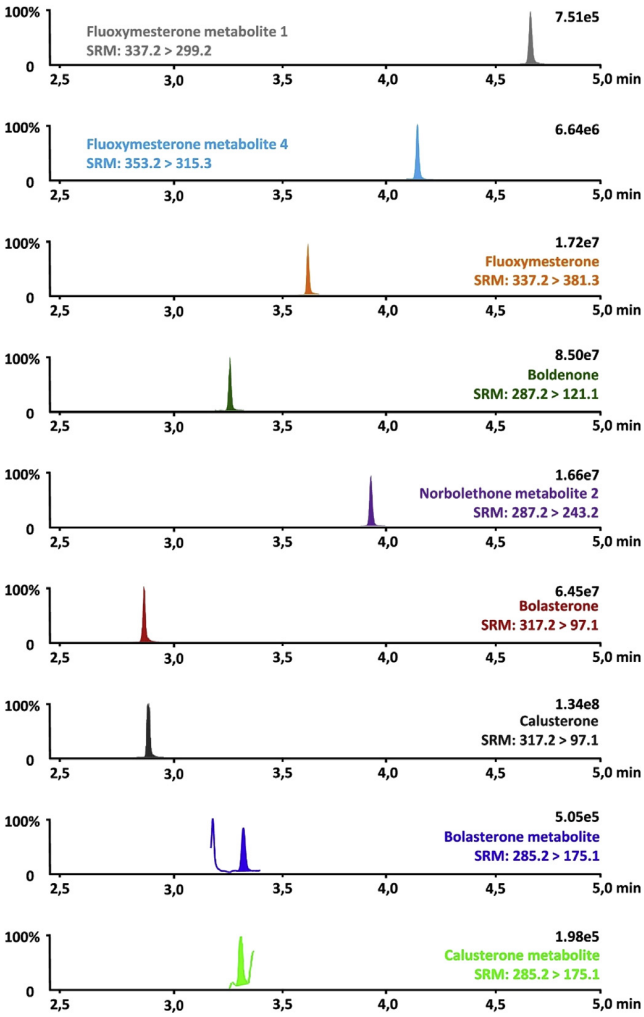

Fig. 4. Chromatograms of nine steroids and related metabolites for injection of urine spiked at 10 ng/mL in UHPSFC-MS/MS.). Reprinted from *J. Chromatogr. A*, Vol. *1451*; V. Desfontaine, L. Novakova, F. Ponzetto, R. Nicoli, M. Saugy, J.L. Veuthey, D. Guillarme; Liquid chromatography and supercritical fluid chromatography as alternative techniques to gas chromatography for the rapid screening of anabolic agents in urine; pp 145–155 [ref 51]. Copyright 2016, with permission from Elsevier.

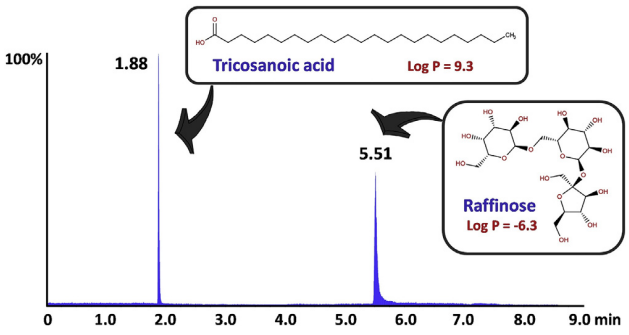

Fig. 5. Chromatogram obtained for the simultaneous injection of tricosanoic acid and raffinose, using acetonitrile/water (50:50) as sample diluent and unified chromatography gradient conditions. Reprinted from *J. Chromatogr. A*, Vol. *1562*; V. Desfontaine, G.L. Losacco, Y. Gagnebin, J. Pezzatti, W.P. Farrell, V. González-Ruiz, S. Rudaz, J.-L. Veuthey, D. Guillarme; Applicability of supercritical fluid chromatography – mass spectrometry to metabolomics. I – Optimization of separation conditions for the simultaneous analysis of hydrophilic and lipophilic substance; pp 96–107 [ref 72]. Copyright 2018, with permission from Elsevier.

In contrast to LC, SFC instruments also offer the possibility to have an online extraction unit linked to the chromatographic system (online SFE-SFC). It is now commercially available and has recently been successfully employed in analytical laboratories in different areas, from the metabolic profiling of drugs metabolites in human urine [38] to the determination of carotenoids and apocarotenoids in human blood [81], and the analysis of polycyclic aromatic hydrocarbons in soil [82]. In these different studies, the authors highlight the very low sample amounts requirement, possibility to achieve fast analysis and how it has been possible to validate those methods [38,81,82]. This type of online SFE-SFC instrument, although it still needs to be more deeply characterized, in particular in terms of connections between the extraction, separation, and detection [82], possesses an impressive potential for the analyses where sample preparation stages can be time-consuming and do not provide sufficient yields.

6. Conclusions

The hyphenation of SFC to MS has undoubtedly known an impressive growth in the last five years. The development of several SFC-MS geometry interfaces has enabled to couple both systems, as well as a discrete handling of the supercritical fluid once the mobile phase is not under the influence of the APBR module. There are, however, still aspects that necessitate to be thoroughly covered to understand the influence of the CO_2 decompression and how to better solve issues related to this phenomenon.

SFC-MS is increasingly being used to analyze compounds present in biological matrices, from urine to plasma, as well as natural substances available in plant extracts. Matrix effects due by biological samples and their impact on the MS signal and performance have been demonstrated to be quite different from what was observed in LC-MS conditions, offering a good complementarity between those two techniques. Nonetheless, there is still a need to further investigate this aspect, with additional sample preparation approaches and different matrices.

ME also impacted sensitivity in a different way than LC-MS, due to the higher probability of signal suppression, rather than enhancement under SFC-MS conditions. Sensitivity, in general, was found to be in several cases at the same level as in LC-MS, if not even higher. However, there are problems related to the limited injection volume and higher dilution factors that, sometimes, make it difficult for SFC-MS to reach LOQ and LOD values obtained in LC-MS.

Finally, the investigation of the most recent applications clearly shows that SFC-MS is moving towards the analysis of small molecules with increasing polarity. This translates in an increasing overlap with RPLC and HILIC. Indeed, the impressive flexibility of SFC in analyzing compounds within an extremely wide polarity range is probably one of the main interests behind this technique. Its complementarity to RPLC drives an increasing number of research groups, and analytical laboratories are starting to use it, to tackle challenging separations achieved under LC-MS conditions.

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Chapter II – Scientific results

II.1 Introduction

The number of scientific articles utilizing UHPSFC besides chiral analysis has increased exponentially over the past ten years. A lot has been done in understanding the fundamentals of this technique, as well as in developing innovative applications with the aim to demonstrate its viability as an alternative to RPLC or HILIC. The implementation of UHPSFC for the analysis of pharmaceuticals, pesticides, compounds derived from natural extracts has been quite successful. In addition, UHPSFC has also begun to be considered in regulated environments such as anti-doping laboratories and pharmaceutical industries.

Nonetheless, there is still a sizable amount of work which is needed to make UHPSFC more attractive as an analytical alternative to LC techniques. Although some investigations on its robustness have started to appear in the past 5 years, the idea that UHPSFC is not able to provide satisfactory results when used for routine analyses is still widespread among scientists. In addition, there are very few examples demonstrating the reproducibility performance of UHPSFC when biological matrices are used.

A second point that needs to be addressed is the applicability range of UHPSFC. It is well established that this technique is adapted to analyze apolar and mildly polar compounds, possibly with small molecular weight (below 1000 Da). The introduction of innovative gradients, such as UC and EFLC, might help SFC to overcome this polarity limit and extend it towards the analysis of polar and highly polar analytes, such as carbohydrates, polar metabolites, and so on. This would translate in UHPSFC overlapping not only with RPLC and NPLC, but also with HILIC.

This chapter will be divided, therefore, into four sections. Initially, the effectiveness of an UHPSFC-MS/MS generic method was evaluated in the context of routine anti-doping analyses. In the second section, some theoretical considerations will be covered regarding the performance of UHPSFC with different percentages of co-solvent, using an UC-type gradient, and how they change at different column temperatures. Finally, in the third and fourth sections, applications of UC-type gradients in UHPSFC-MS in the fields of metabolomics and peptide analysis will be discussed.

II.2 Implementation of UHPSFC-MS/MS in routine anti-doping laboratories

II.2.1 Estimation of retention times variability under reproducible conditions

The stability of retention times in UHPSFC has often been a problematic feature of UHPSFC-based analytical methods. Indeed, due to the relatively high compressibility of the mobile phase, the generation of density gradients was often observed. These would impact the elution strength of the mobile phase, thus contributing to the poor reproducibility of the retention profiles and, therefore, the retention times.

In recent years, manufactures have greatly improved the instrumentation, with the specific purpose to reduce as much as possible this phenomenon. Moreover, as higher co-solvent percentages have begun to be systematically used in UHPSFC, an improvement of the method robustness is expected, as the liquid component of the mobile phase is increased. Finally, new columns, dedicated for UHPSFC analyses, have been released on the market, aiming in reducing as much as possible the known formation of silyl ether groups (SEF) on the surface of the silica particles.

In this article, the focus was put on how the combined addition of water in the mobile phase, together with the use of the latest generation of UHPSFC columns (Torus 2-PIC) has enabled UHPSFC-MS/MS to generate similar performance to UHPLC-MS/MS in terms of retention times reproducibility over a period of four months, using a library of 57 doping agents in neat solutions and spiked in diluted urine.

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Supercritical fluid chromatography–mass spectrometry in routine anti-doping analyses: Estimation of retention time variability under reproducible conditions

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ABSTRACT

The aim of this study was to estimate the retention time variability under reproducible conditions of an SFC-MS analytical method for routine anti-doping analyses. For this purpose, a set of 51 doping agents, as neat standards and spiked in diluted urine, was used to assess their retention times variability over a period of four months, as well as the column *inter-batch* reproducibility. Three UHPSFC stationary phases have been employed, the Acquity UPC^2 Torus 2-Picolylamine (2-PIC), UPC^2 Viridis BEH and Acquity UPLC HSS C18 SB. Four columns, per column chemistry, have been purchased to represent three different production lots, with a total of twelve columns employed in this study. The two columns from the same lot were applied to the first part of the study (repeatability), whereas the representative of three different lots were employed in the second part (robustness). In terms of organic modifier, a mixture of 98% MeOH and 2% water containing 20 mM ammonium formate was selected in order to limit the formation of methyl-silyl ethers on the surface of the silica particles, thus potentially improving the repeatability of retention times. A comparison with an UHPLC reference analytical method was made with the same set of analytes.

The average relative standard deviations (RSD%), represented in split violin plots, illustrate how two of the UHPSFC columns assessed in this study were able to generate an excellent repeatability of retention times, with results that are in a similar range of those generated by UHPLC. Moreover, the Torus 2-PIC has proven to be the best of the three stationary phases, with an impressive RSD% of 0.5% in diluted urine relative to the *inter-month* variability. Finally, the *inter-batch* reproducibility assessment has highlighted a good reproducibility of the same stationary phase belonging to different production lots for all three column chemistries assessed, with the Viridis BEH silica generating an RSD% of 0.7% in diluted urine. Higher values of RSD (%) were found for Torus 2-PIC and HSS C18 SB, respectively of 1.0% and 1.6%.

1. Introduction

The history of anti-doping analysis in sport has some deep roots, due to the widespread phenomenon of doping practices by several athletes. In 1999, the World Anti-Doping Agency (WADA) has been created to fight against doping, with the clear aim to ensure high quality anti-doping programs and regulations worldwide, as well as to standardize the analytical techniques and performance of the different accredited anti-doping laboratories [1]. The task has been proven to be, however, extremely challenging. Every year new doping agents are identified and included in the list of prohibited substances published by the WADA [2]. The listed compounds belong to different chemical categories, from *e.g.* anabolic agents and peptide hormones to β2-agonists, diuretics, stimulants, cannabinoids among others. Screening these heterogeneous classes of compounds can impose several challenges to method development, especially as tests are performed on

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biological matrices, mainly urine samples. In order to perform reliable analyses, a lot of efforts have been put on the development of reliable and robust analytical techniques, as well as proper sample preparation procedures, which enable anti-doping laboratories to reach the Minimum Required Performance Levels (MRPLs) established by WADA [3]. Currently, the state-of-the-art techniques in anti-doping analysis are mainly gas chromatography (GC) [4–6] and ultra-high performance liquid chromatography (UHPLC) [7–9], coupled to tandem mass spectrometry (MS/MS) or high resolution mass spectrometry (HRMS). The analyses are combined with an extensive selection of sample preparation techniques such as liquid-liquid extraction (LLE) [10], solid-phase extraction (SPE) [11], or simple dilute-and-shoot (DS) and protein precipitation (PP) [12]. Nonetheless, there is a constant need to develop novel analytical techniques providing enhanced performance, faster response time delivery, higher sensitivity and, when possible, orthogonal separation profile to the already existing platforms.

Supercritical fluid chromatography (SFC) has experienced a remarkable growth in the last decade, due to the commercialization of a new generation of instruments, allowing SFC to be considered as ultra-high performance SFC (UHPSFC) [13]. The attractiveness of SFC is based on the use of a supercritical mobile phase, where the combined low viscosity and high solvating power represent a clear advantage, in terms of kinetics and selectivity over UHPLC and GC [14]. However, the use of a super/subcritical mobile phase can be challenging for the repeatability of an SFC analytical method. the high compressibility of a supercritical fluid causes, even with slight change in backpressure, density gradients in the mobile phase [15–17]. These density gradients have proven to be quite deleterious for the robustness of SFC methods, as they affect the elution power of the mobile phase and, therefore, cause retention time shifts and selectivity modifications [15–17]. Moreover, the use of biological matrices can add more variability to SFC methods, as polar compounds coming from the biological matrix might strongly adhere on the stationary phase, which often possesses polar characteristics, causing a change in retention and selectivity. Due to these reasons, until today, SFC was almost never considered as a valid alternative to LC or GC in highly regulated fields such as anti-doping or forensic routine analyses.

Despite these issues, the implementation of more robust UHPSFC instruments has opened new possibilities. Indeed, there have been several recent studies demonstrating the applicability of UHPSFC in domains where stringent requirements in terms of sensitivity and selectivity are necessary [18]. As an example, the potential use of UHPSFC, coupled to tandem mass spectrometers (MS/MS), in doping control analyses was successfully evaluated [19–23]. Moreover, it was demonstrated that UHPSFC-MS/MS successfully meets the sensitivity limits and the MRPLs for the majority of classes of prohibited compounds required for anti-doping analyses in biological matrices [24,25]. Besides the sensitivity aspect, there is also still a lot to do to demonstrate the robustness and stability of UHPSFC-MS/MS methods, in particular when evaluating the presence of prohibited doping substances in a complex biological matrix such as human urine. Very few articles are present in the literature, related to the potential implementation of SFC-MS/MS in routine applications [26,27]. In such works, however, the authors have assessed the robustness of the developed methods only over a relatively short timeframe.

The aim of this study was, therefore, to evaluate retention time stability of target compounds in UHPSFC-MS/MS conditions over a period of four months for a set of 51 doping agents, in aqueous-organic solution, and in human urine samples. The intra-day, inter-day, inter-week and inter-months repeatability was assessed. Moreover, the inter-batch column variability was also evaluated. Finally, a comparison was made with the current state-of-the-art UHPLC-MS/MS approach for the same doping agents.

2. Experimental section

2.1. Chemicals and reagents

Reference neat standards of doping agents, including timolol, atenolol, propranolol, ethisterone, fluoxymesterone, gestrinone, oxandrolone, stanozolol, 3'-hydroxystanozolol, salbutamol, formoterol, terbutaline, letrozole, tamoxifene, clomiphene, 4-hydroxyclomiphene, trimetazidine, furosemide, amiloride, bendroflumethiazide, hydrochlorthiazide, eplerenone, chlortalidone, probenecide, bumetanide, etacrynic acid, cocaine, etilephrine, oxilophrine, octopamine, niketamide, *N*-ethylnicotinamide, methylphenidate, ritalinic acid, metamphetamine, fenfluramine, amphetamine, furfenorex, fenbutrazate, benzylpiperazine, isometheptene, 6-OH-bromantan, morphine, fentanyl, norfentanyl, JWH-250 metabolite 1 and 2, betamethasone, dexamethasone, prednisone and prednisolone (Table S1) were kindly provided by the Swiss Laboratory for Doping Analyses (Epalinges, Switzerland).

Methanol (MeOH), acetonitrile (ACN) and water LC–MS grade were purchased from Fisher Scientific (Loughborough, UK). Ammonium formate (AmF) was purchased from Sigma-Aldrich (Buchs, Switzerland). Pressurized carbon dioxide (CO_2) of 3.0 grade (99.9%) was purchased from PanGas (Dagmerstellen, Switzerland).

2.2. Standard solutions

Stock neat standard solutions of each doping agent were prepared in MeOH at a concentration of 1 mg/mL. Six mixtures of reference neat standards (five mixtures for positive ESI, and one mixture for negative ESI), at three different concentration levels (200 ng/mL–2 µg/mL–20 µg/mL), were prepared by diluting stock neat standard solutions with water/ACN 25/75 v/v. A further dilution, to final concentrations of 10 ng/mL-100 ng/mL-1 µg/mL, was made with the same solvent (Table S2).

2.3. Biological samples and sample treatment

Urine samples were prepared according to a dilute-and-shoot (DS) approach, using a mixture of water/ACN 25/75 v/v as sample diluent solvent. The choice of this solvent was based on a previous work [25]. Six urine samples, obtained from three healthy men and three healthy women volunteers, were mixed to obtain a representative urine sample pool. The pooled urine was first centrifuged at 3000*g* for 6 min, then the supernatant was collected and filtered through a 0.45 µm nylon membrane.

The filtered pooled urine was then divided into six aliquots (100 µL each), spiked in singlets with 50 µL of the reference standard mixtures and with 50 µL of mefruside (internal standard) at 1 µg/mL. Aliquots were diluted further with water/ACN 25/75 v/v to a volume of 1000 µL to obtain the final diluted urine solutions. The final concentration of mefruside (internal standard) was set at 50 ng/mL.

2.4. Column chemistries

Three different SFC stationary phases, purchased from Waters (Milford, MA, USA), have been selected: Acquity UPC^2 Torus 2-Picolylamine (2-PIC), UPC^2 Viridis BEH and Acquity UPLC HSS C18 SB. All these columns have dimensions of 100 × 3.0 mm I.D. and particle size of 1.7 µm, except the HSS C18 SB, which has 1.8 µm particle size. For each column chemistry, four columns were purchased. In total, the four columns represented three different production lots. The two columns from the same lot were applied to the first part of the study (repeatability), whereas the representative of three different lots were employed in the second part (robustness).

2.5. UHPSFC-MS/MS analysis

All experiments were performed on a Waters Acquity UPC^2 system, equipped with a binary solvent delivery pump, an autosampler, a column oven, a UV detector fitted with an 8 µL flow-cell and a two-step (active + passive) backpressure regulator (BPR). The Waters Acquity UPC^2 system was hyphenated to a Waters TQD Triple Quadrupole mass spectrometer, fitted with a Z-spray ESI source. The hyphenation between the chromatographic system and the triple quadrupole detector was made via a dedicated double-T splitter interface from Waters. Additional make-up solvent (pure MeOH at a flow-rate of 0.3 mL/min) was brought by a Waters Isocratic Solvent Manager (ISM) pump.

The TQD detector operated in positive and negative ESI modes, with Selected Reaction Monitoring (SRM) acquisition mode. The mixtures were systematically analyzed in only one single ionization mode (ESI+ or ESI-), without requiring any polarity switching. ESI modes for each mixture analyzed have been specified in supplementary Table S2. The source temperature was set at 140 °C. Nitrogen was used as the desolvation gas at 900 L/h and 450 °C, and as the cone gas at 100 L/h. The collision gas was argon. The capillary voltage was set at ±1.0 kV. Individual values for transitions, cone voltages and collision energies are listed in Table S3 of the Supplementary material. Dwell times were set at 20 ms for ESI positive mixtures, while a dwell time of 25 ms was chosen for the ESI negative mixture.

The mobile phase was composed of a mixture of CO_2 and 20 mM AmF in 98/2 v/v MeOH/water as the modifier, delivered with a flow-rate of 1.20 mL/min. The gradient profile was as follows: isocratic step of one minute at 2% modifier, then a ramp from 2% to 50% in four minutes, followed by a second isocratic step at 50% modifier for one minute, initial conditions were then kept for a total run time of seven minutes. The column temperature was set at 40 °C, while the BPR pressure was 12 MPa. The autosampler temperature was set at 8 °C and injection volume was 2 µL. Pure MeOH was used as both weak and strong wash solvents, with their volumes being 600 µL and 200 µL, respectively. Data acquisition and instrument control were performed with MassLynx 4.1, while data processing was performed with TargetLynx 4.1.

2.6. Sequence structure

A specific sequence structure (or series) was adopted to collect all the necessary data for the purposes of this study (Fig. S1a). Each mixture was injected three subsequent times; once all mixtures were analyzed, a waiting period of two hours would follow, with a reduced flow of 0.50 mL/min of mobile phase (98:2 CO_2/B; B: 20 mM AmF in 98/2 v/v MeOH/water) going through the column. After two hours, the same analysis was performed, followed by a second waiting period and, finally, by a third analysis cycle. Such sequence was repeated on each column, for standard and diluted urine samples, over a total period of 16 weeks–4 months (Fig. S1-b).

A similar approach was utilized for the *inter-batch* variability assessment (Fig. S2), with the analysis spread over a period of 3 weeks.

2.7. Data treatment

Variations of retention time were systematically investigated by implementing balanced experimental designs involving three levels for neat neat standards solution, and four levels in urine. A hierarchical decomposition of variance was carried out using nested Analysis of Variance (ANOVA) to evaluate within-day repeatability, as well as between-day, between-week (neat neat standards solution and urine) and between-batch effects (urine only). In the spirit of validation of bioanalytical methods, this strategy involved a principle of summation to compute variance estimates: intra-day variance was related to within-day repeatability, the sum of intra- and inter-day variances lead to between-day variability, the sum of intra-day, inter-day and inter-week variances provided between-week differences, etc. Thanks to the balanced designs used and the absence of any missing values, accurate least-squares estimates could be obtained. Due to experimental constraints inter-month variance was estimated separately using triplicates (neat standards solution and urine). Finally, all the results were reported as relative standard deviation (RSD) of the retention times. All calculations were performed in the MATLAB® 9.5 environment (The MathWorks, Natick, USA) using the *anovan* function with the 'nested' option.

2.8. Split violin plot interpretation

The calculated RSD values were plotted as split violin plots. Violin plots can graphically represent the data distribution of a population of data by combining a box plot and a rotated density plot (Fig. S3). Inspecting the shape of the violin plot representing the density estimate of the data points allows regions with a higher frequency of finding particular values to be easily highlighted.

In this study, split violin plots have been employed to have a systematic comparison of RSD values found with neat standards *vs* diluted urine samples *per* stationary phase. Within a split violin plot, the left side represents the population data relative to neat standards solution, while the right side is for diluted urine samples. Split violin plots were created using Plotly Chart Studio (https://chart-studio.plot.ly).

3. Results and discussion

3.1. Intra-day variability

The initial step in the assessment of retention times variability under UHPSFC-MS conditions consisted in its evaluation over a short time frame (*intra-day*), corresponding to the repeatability of the same injection within the same sequence. To avoid any influence of column inter-batch variability, columns with identical stationary phase belonging to the same batch were exclusively employed. The average RSDs relative to the *intra-day* variability are displayed in Table 1 for each tested column. To have a more comprehensive view of the data set collected and calculated, split violin plots (Fig. 1) were built, allowing a direct comparison between the two matrices (neat standard solutions *vs* diluted urine) and the employed columns. Using these graphical representations and the values depicted in Table 1, different trends can be observed: the *intra-day* variability of the three columns was almost identical, with average RSD values (%) in the range 0.14–0.19% for standard and urine samples. Such values are relatively low, illustrating that the developed analytical method, was able to offer an excellent performance between immediate injections. Although average RSD values were quite similar to each other, there was a particularity of the Torus 2-PIC, to present more outliers in comparison to the other stationary phases. This phenomenon, however, can be quite easily explained by looking at the average retention times for each column and by comparing them with the gradient profile being used (Fig. 2). Fig. 2 shows that the retention of at least 4 analytes (i.e. furferonex, cocaine, fenbutrazate and niketamide) was quite limited on the Torus 2-PIC, while there was a strong retention of all doping agents on the Viridis BEH silica and HSS C18 SB, with an elution window between 20% and 50% of co-solvent. The poor retention of those four analytes, whose elution requires a small amount of co-solvent (2-10%), falls in an area where the mobile phase is more subjected to possible density gradients,

Table 1
Average relative standard deviations (RSD%) values, for the three UHPSFC stationary phases evaluated in this study, obtained with neat standards and diluted urine.

	BEH silica-STDs	BEH silica-URINE	Torus 2-PIC-STDs	Torus2-PIC-URINE	HSS C18 SB-STDs	HSS C18 SB-URINE
Average Intra-day	0.2%	0.1%	0.2%	0.2%	0.1%	0.1%
Average Inter-day	0.2%	0.4%	0.2%	0.2%	0.5%	0.6%
Average Inter-week	1.0%	0.7%	0.3%	0.6%	1.2%	1.3%
Average Inter-month	1.5%	1.3%	0.3%	0.5%	1.5%	5.3%
Average Inter-batch	-	0.7%	-	1.0%	-	1.6%

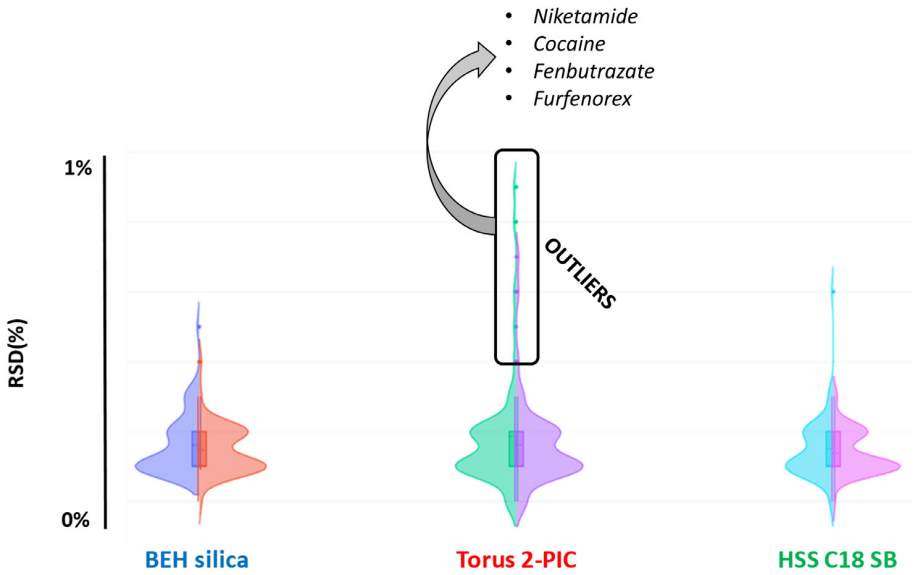

Fig. 1. Split violin plots of relative standard deviations (RSD%) values, relative to the *intra-day* variability of retention times, for the three UHPSFC stationary phases evaluated in this study. Left side of each violin plot: RSD values obtained with neat standard solutions. Right side of each violin plot: RSD values obtained with diluted urine.

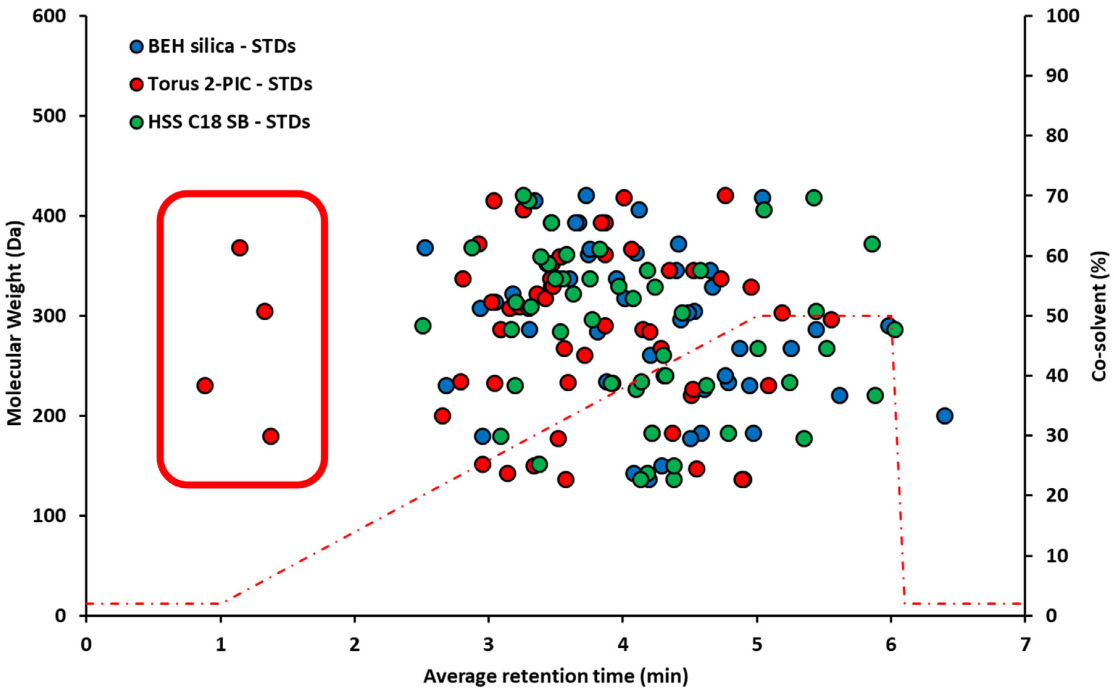

Fig. 2. Distribution of average retention times, obtained on each column over the total period of analysis (4 months), *vs* gradient slope and *vs* molecular weight.

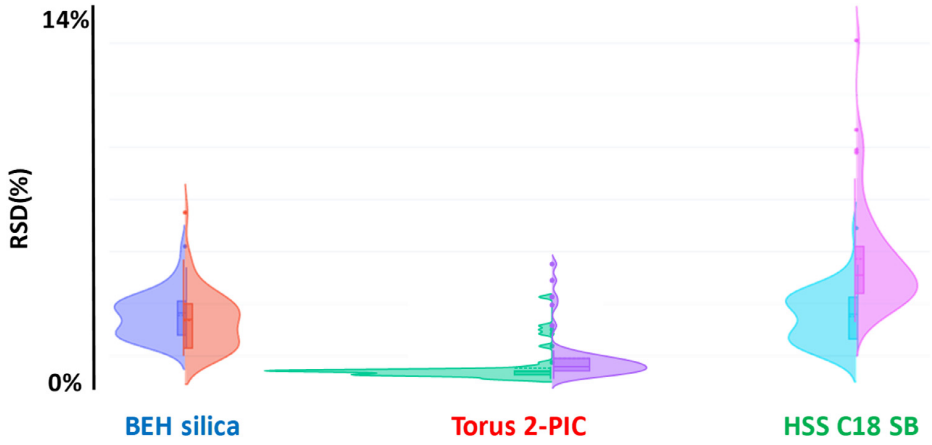

Fig. 3. Split violin plots of relative standard deviations (RSD%) values, relative to the *inter-month* variability of retention times, for the three UHPSFC stationary phases evaluated in this study.

therefore translating in a potentially higher but still acceptable (i.e. below 1%) variability of retention times.

3.2. Inter-day, inter-week and inter-month variability

3.2.1. Impact of the surface chemistry

Moving on from the *intra-day* to the *inter-day, inter-week* and, finally, *inter-month* variability (Table 1), the assessment of the three stationary phases provided more notable differences. A classification between the three stationary phases was noticed, especially when considering the *inter-month* period of 16 weeks – 4 months (Fig. 3). Over the 16 weeks of analysis, the HSS C18 SB clearly appeared as the lowest-performing column in terms of repeatability, for both neat standard solutions and, more importantly, diluted urine, with an average RSD of up to 5.3% (Table 1). Regarding the two other stationary phases being tested, the Viridis BEH silica presents a lower repeatability (Fig. 5), but the average RSD values within this period were well below 2% in both matrices (Table 1), indicating a good stability of retention times. The Torus 2-PIC, on the other hand, was demonstrated to offer a very low variability in retention of target compounds, with RSD values of 0.3% (neat standards) and 0.6% (diluted urine) over the period of 16 weeks.

A credible explanation of such differences revolves around the type of silica particle chosen by the manufacturer, as well as the chemistry of the selector. Among the tested columns, the Viridis BEH silica and Torus 2-PIC are composed of, respectively, first and second generation of silica particles developed exclusively for SFC analyses. They both belong to the family of hybrid silica particles that has demonstrated better performance and robustness than classic silica under UHPLC conditions. Because no selector is attached to the surface of the particles of the Viridis BEH silica, the retention is exclusively governed by the presence of free silanols. The Torus 2-PIC, on the other hand, undergoes a two-stage functionalization, with the aim to minimize the presence of surface silanols on the silica particles and ensure the highest coverage of the particle with the 2-pycolamine group, thus reducing the impact of secondary interactions. Therefore, the main difference between these two columns is the presence (or absence) of the selector. The obtained results clearly point out how both columns provided good results with a low retention times variability (Table 1). Nonetheless, it seems that the 2-pycolamine group, is capable of generating a wider range of interactions with the analytes, for example H-bonds as well as some π-π interactions. Moreover, there could be an adsorption phenomenon of water (which is used as a mobile phase additive) onto the stationary phase, which can enhance some specific interactions offering a more controlled and reproducible retention profile with the experimental conditions being employed, leading to an enhanced stability of retention times.

The HSS C18 SB, on the other hand, possesses some major differences with the other stationary phases chosen for this study. Indeed, this column has not been originally developed for UHPSFC but for UHPLC applications. Moreover, on this stationary phase, no endcapping procedure was employed, leaving therefore a relatively large amount of underivatized silanols at the surface of the silica particles, to offer a higher degree of orthogonality in comparison to other UHPLC C18 columns available on the market. Under UHPSFC conditions, the residual silanols are used to create retention mostly through H-bonding interactions, while the C18 group is responsible mainly of secondary interactions. This was demonstrated by West *et al.* a few years ago, as this column was ranked as a polar stationary phase under UHPSFC conditions [28]. However, the accessibility of silanols on the HSS C18 SB phase seems hindered by the presence of the C_{18} alkyl chain in a randomized way. Finally, on this stationary phase, the co-solvent adsorption phenomenon should not happen in the same way as on the Torus 2-PIC column, due to the presence of apolar C_{18} functional group. The presence of the selector can therefore be considered as highly detrimental in terms of retention times stability, especially over a prolonged period of time such as the one considered in this study. Moreover,

3.2.2. Formation of methyl-silyl ethers

The possible formation of methyl-silyl ethers, over time and under SFC conditions, has been already reported [29]. the presence of small alcohols such as methanol in the mobile phase (the most employed polar organic modifier in SFC analytical methods), can induce the methylation of free silanols present on the silica surface of SFC columns [29]. Due to this chemical modification, polar interactions taking place in SFC between analytes and silanols can be strongly reduced over extended time, leading to reduction of retention times and poor repeatability. Obviously, this phenomenon concerns columns with the highest number and most accessible silanols. In the present study, this is the case of bare silica stationary phase (Viridis BEH silica), and to a lesser extent, the HSS C18 SB. On the third column (Torus 2-PIC), the procedures employed by the column manufacturer to reduce the presence and accessibility of silanols should be sufficiently effective in preventing such phenomenon. Quite interestingly, the same authors have also reported that the addition of water in the mobile phase, even at very low amounts as those generally employed in UHPSFC, could be beneficial in avoiding the methylation to happen [29].

Therefore, it was decided to implement the addition of water in limited concentrations (2% v/v in the organic modifier), with

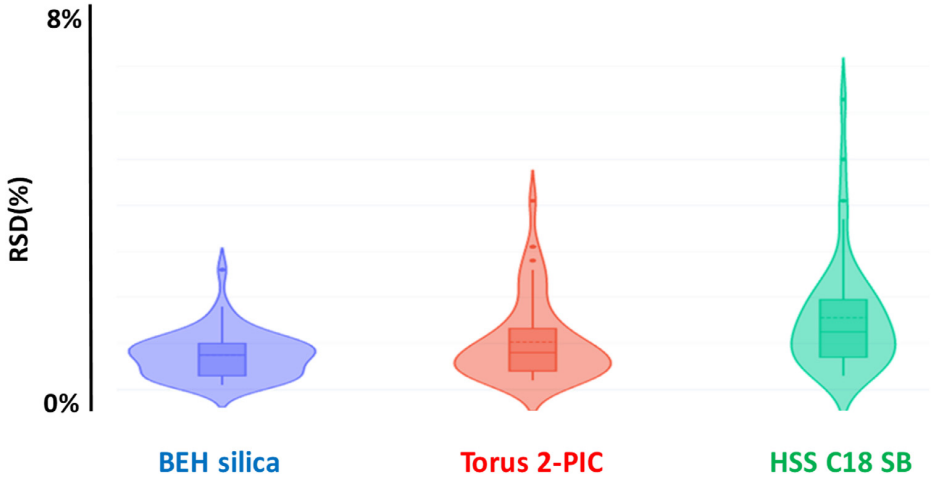

Fig. 4. Violin plots of relative standard deviations (RSD%) values, relative to the *inter-batch* variability of retention times, for the three UHPSFC stationary phases evaluated in this study.

the hypothesis that it would be beneficial in avoiding, or keeping to a minimum level, such methylation phenomenon. The analysis of the split violin plots relative to the *inter-month* retention times variabilities (Fig. 3) has highlighted how columns with a moderate or important presence of free silanols are those generating the highest variability. The overall performance was quite good on the Viridis BEH silica, where the repeatability over a period of four months provided an RSD value of about 1.6% for neat standards and 1.4% for urine (Table 1). These good results observed on Viridis BEH silica and Torus 2-PIC can be considered an additional demonstration of how water, even as an additive in the co-solvent (2% in MeOH), can have a significant impact on keeping the retention times variability extremely low by efficiently demethylating silyl-ethers, but this is not valid for all stationary phases.

3.3. Inter-batch variability

When performing an analysis in a routine environment, it is also mandatory to control the transferability of the developed method among different laboratories. To do so, one must firstly check that different batches of the same stationary phase provide identical performance and values of retention times. Therefore, to give a more comprehensive view on the robustness of retention times under UHPSFC-MS conditions, the contribution of the column *inter-batch* variability has been also investigated. A total of three columns *per* column chemistry, all with different batch numbers, have been tested over a total period of three weeks. At this point of the study, the use of neat standard solutions was not deemed useful, since real-life routine analyses are mostly performed on biological matrices, namely diluted urine. As shown in Fig. 4, violin plots have been generated to have a graphical representation of this data set, similarly to what has been previously done. Their assessment marks, once more, a very low variability of retention times for the doping agents being tested. The average values of RSD were all well below 2% on all stationary phases (Table 2), and in two cases (Viridis BEH silica and Torus 2-PIC) the average RSD values were approximately equal or even lower than 1%. However, a different ranking of the three stationary phases has been observed. the column generating the lowest variability is not the Torus 2-PIC column, but the Viridis BEH silica, while HSS C18 SB still provides the worst results among the three columns being evaluated.

When assessing the *inter-batch* variability, different factors have to be taken into consideration, namely the reproducibility of the column packing, and the silica pre-treatment and functionalization. Taken together, these three factors can explain some differences observed between the variability over time and shifts resulting from different column batches. The Viridis BEH silica is indeed giving the best performance because it does not undergo the silica functionalization procedure. On the contrary, the HSS C18 SB and Torus 2-PIC undergo such treatment. However, there are some major differences between the HSS C18 SB and the Torus 2-PIC. As already discussed, the retention under UHPSFC conditions is not mainly generated by the C_{18} bonding on the HSS C18 SB, but rather by the presence of underivatized silanols. The Torus 2-PIC, however, possesses a selector that assures retention of analytes. It is important to notice that the column provider decided to implement an innovative functionalization strategy on the Torus 2-PIC, consisting of a two-stage reaction procedure to minimize secondary interactions and ensure an optimal coverage of the silica particles with the selectors. Unfortunately, such procedure is still claimed to be under patenting by the column manufacturer, so there is not much that can be disclosed at this point. Nonetheless, this innovative approach seems to provide some advantages,

Table 2
Average relative standard deviations (RSD%) values, for the three UHPSFC stationary phases and the UHPLC stationary phase evaluated in this study, obtained with diluted urine.

	BEH silica - URINE	Torus 2-PIC - URINE	HSS C18 SB - URINE	UHPLC C18 - URINE
Average Inter-day	0.1%	0.2%	0.1%	0.1%
Average Inter-day	0.4%	0.2%	0.6%	-
Average Inter-week	0.7%	0.6%	1.3%	0.4%
Average Inter-month	1.3%	0.5%	5.3%	0.8%
Average Inter-batch	0.7%	1.0%	1.6%	-

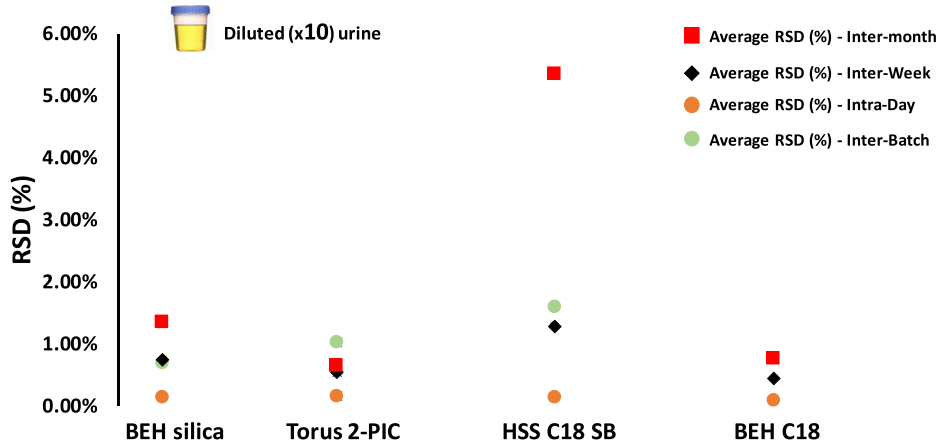

Fig. 5. Comparison of average RSD (%) values, obtained in diluted urine, of the three UHPSFC stationary phases *vs* UHPLC C18 stationary phase.

as the violin plots (Fig. 4) and the average RSD values (Table 2) were much better on the Torus 2-PIC *vs.* HSS C18 SB.

3.4. Comparison with UHPLC-MS/MS

After successfully investigating the retention time variability in SFC over a predetermined period of time, or shifts generated by the use of columns from different batches, a systematic comparison against the state-of-the-art analytical techniques being used in routine anti-doping laboratories is needed. To do so, retention time values for the same set of doping agents being assessed with UHPSFC-MS/MS have also been similarly evaluated for UHPLC-MS/MS, over the same period (16 weeks). The average RSD values obtained, for UHPLC-MS/MS, were extremely low with values below 1.0% (Table 2), indicating an excellent repeatability of the analytical method. It is, however, interesting to compare these values with those for UHPSFC-MS/MS. The average RSD values for all UHPSFC columns and for the reference UHPLC column employed in routine (Acquity BEH C18) are displayed in Fig. 5. This comparison provides some interesting results: one UHPSFC column (HSS C18 SB) is simply unable of generating the same performance as those obtained with UHPLC-MS/MS, with an RSD of 5.4% for the *inter-month* variability vs an RSD of about 0.8% over the same period under UHPLC conditions. The two other SFC column chemistries, however, provided much closer values to UHPLC results. Specifically, average *inter-month* RSD of only 0.5% and 1.3% were obtained for the Torus 2-PIC column and Viridis BEH silica, respectively, and these values were comparable to those obtained under UHPLC-MS/MS conditions. These results clearly point out that it is possible nowadays to reach almost an equivalent performance between UHPSFC-MS/MS and UHPLC-MS/MS in terms of retention time stability.

4. Conclusions

In the present study, an assessment of the retention time stability under UHPSFC-MS/MS conditions was performed, with a comparison against the state-of-the-art analytical technique employed in routine anti-doping laboratories (UHPLC-MS/MS). The results obtained in neat standards solution and diluted urine, pointed out their equivalence, in terms of retention stability performance, over a period of four months with two out of the three UHPSFC stationary phases evaluated. It was also established how the *inter-batch* variability was fully acceptable in most of the tested conditions. The results reported in this study confirm that the identification criteria set for retention times in qualitative chromatographic-mass spectrometric methods can be applied also to the method based on UHPSFC-MS/MS. These results also indicate that great progresses have been made to establish modern UHPSFC-MS/MS as a reliable and robust analytical technique, with the potential of being employed also in routine laboratories.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2019.460780.

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II.2.3 Assessment of the interlaboratory reproducibility with urine samples

Although the robustness of the retention times was assessed, the analyses have been performed in a single laboratory. In order to ensure that a method is truly reproducible, its variability between different laboratories must be evaluated. Very few works have been done on this subject, and to the knowledge of the author, none has been done with biological matrices. It is, therefore, extremely important to verify how UHPSFC-MS/MS performs in different laboratories when analyzing substances in biological matrices, especially if its implementation in anti-doping routine laboratories is being questioned.

In this second work, an interlaboratory study was conducted among four laboratories: one in Germany (Waters DemoLab – Eschborn); one in France (LABERCA – Nantes); one in Czech Republic (Charles University - Hradec Králové) and one in Switzerland (Swiss Laboratory for Anti-Doping Analyses – Lausanne). The performance of the same UHPSFC-MS/MS method, using the same chromatographic system (Waters Acquity UPC^2), have been tested with focus on the retention times stability, using a set of 21 doping agents. In addition, each laboratory tested a set of seven blind urines, in which up to two unknown doping agents were present, with the developed UHPSFC-MS/MS method.

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COMMUNICATION

Ultra-high performance supercritical fluid chromatography coupled to tandem mass spectrometry for antidoping analyses: Assessment of the inter-laboratory reproducibility with urine samples

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Abstract

The aim of this study was to assess the interlaboratory reproducibility of ultra-high performance supercritical fluid chromatography coupled with tandem mass spectrometry method for routine antidoping analyses. To do so, a set of 21 doping agents, spiked in urine and analyzed after dilute and shoot treatment, was used to assess the variability of their retention times between four different laboratories, all equipped with the same chromatographic system and with the same ultra-high performance supercritical fluid chromatography stationary phase chemistry. The average relative standard deviations (RSD%) demonstrated a good reproducibility of the retention times for 19 out of 21 analytes, with RSD% values below 3.0%. Only for two substances, namely fenbutrazate and niketamide, the retention was not repeatable between laboratories, with RSD% of approximately 15% in both cases. This behaviour was associated with (a) the low organic modifier percentage (around 2-4%) in the mobile phase at the corresponding retention times, and (b) the influence of the system volume on poorly retained analytes. An analysis on seven "blind" urines was subsequently carried out in the same four laboratories. In these blind samples, either one, two, or none of the 21 doping agents previously analyzed were present at an unknown concentration. Each laboratory had to perform the identification of the compounds in the samples and estimate their concentrations. All laboratories assigned all target analytes correctly in all blind urine samples and provide a comparable estimation of their concentrations.

KEYWORDS
anti-doping analyses, inter-laboratory reproducibility, tandem mass spectrometry, ultra high-performance supercritical fluid chromatography

1 | INTRODUCTION

The world of antidoping analyses is in constant evolution, as more strict criteria are regularly defined by the World Anti-Doping Agency (WADA) to promote clean sport and enhance the deterrence from doping practices. Therefore, a lot of emphases is put on the improvement of analytical techniques already employed in routine anti-doping laboratories.[1–3] Moreover, new analytical approaches are also considered, with the potential of additional advantages to the analysts such as faster time analysis and improved throughput. In this context, the implementation of ultra-high performance supercritical fluid chromatography, coupled to tandem mass spectrometry (UHPSFC-MS/MS), has received a great deal of interest in the last few years from different research groups and antidoping laboratories throughout the world.[4–8] Thanks to its unique separation profile, complementary to that achievable with reversed-phase liquid chromatography (RPLC) and excellent kinetic performance due to the use of supercritical fluid in the mobile phase, UHPSFC-MS/MS can indeed be successfully employed with challenging samples, providing similar or even better results than ultra-high-performance liquid chromatography (UHPLC) coupled to MS/MS systems.[9–11] Furthermore, UHPSFC-MS/MS does not pose the same challenges related to sample preparation as gas chromatography (GC), as it does not require any derivatization step prior to analysis. One of the historical issues with SFC was its scarce method robustness.[12–14] The use of mobile phases with limited percentages of cosolvent (<10-15%), in combination with the limited capability of the pumping system in handling supercritical fluids, has often translated, in the past, into poorly robust methods. This was due to the high compressibility of the mobile phase itself, as well as from the formation of density gradients throughout the column. Moreover, the instrumentation was not able to perform rugged analyses and quantitative performance was always poor. However, the shift towards UHPSFC, allowed by the introduction of a new generation of instruments in 2012, seems to have successfully adressed this challenge. In the past 3-5 years, there has been an increasing number of studies focusing on how UHPSFC can guarantee similar performance to UHPLC in terms of method robustness. The comparison has been demonstrated with standard compounds and, more recently, with biological matrices too.[8,15,16] Nonetheless, nothing has been done so far in assessing the robustness of a UHPSFC bioanalytical method across different laboratories. This point is of vital importance if UHPSFC has to be considered a viable option for routine analyses in regulated environments.

The aim of this work was to assess the retention times variability of a UHPSFC-MS/MS method across four different laboratories, using a set of 21 doping agents spiked, at two different concentration levels, in urine treated following the dilute-and-shoot (DS) procedure. Secondly, an evaluation of the performance of such UHPSFC-MS/MS method in analyzing seven different blind urine samples in the same four laboratories was carried out.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Reference doping agents, namely amiloride, amphetamine, atenolol, cocaine metabolite (benzoylecgonine), fenbutrazate, fentanyl, fentanyl metabolite (norfentanyl), fluoxymesterone, gestrinone, hydrochlorothiazide, JWH 250 metabolite (JWH-N-(5-carboxypentyl)), niketamide, niketamide metabolite (N-ethylnicotinamide), prednisone, probenecide, propranolol, salbutamol, stanozolol, tamoxifene, terbutaline, trimetazidine, and one internal standard (salbutamol-*d5*) were kindly provided by the Swiss Laboratory for Doping Analyses (Epalinges, Switzerland).

The minimal quality level for solvents and reagents in each laboratory were: methanol (MeOH), acetonitrile (ACN), and water (H_2O) of LC-MS grade and ammonium formate (AmF) at 99.9% purity level. Pressurized carbon dioxide (CO_2) of at least 3.0 grade (99.9%) was employed in each laboratory.

2.2 | Standard solutions and biological samples treatment

Stock solutions of each doping agent were prepared in MeOH at a concentration of 1 mg/mL. From these solutions, two stock solutions containing all 21 doping agents at either 500 or 50 ng/mL and 50 or 5 ng/mL were prepared in ACN/H_2O 75:25 v/v (Table S1).

Urine samples have been prepared using a dilute-and-shoot (DS) procedure, with a mixture of ACN/H2O 75:25 *v/v* as sample diluent. The choice of this solvent was based on a previous work.[9] The DS procedure is as follows: six urine samples, obtained from three healthy men and three healthy women volunteers, were mixed to obtain a representative urine sample pool. The pooled urine was filtered through a 0.22 µm nylon membrane. Subsequently, two aliquots of 100 µL of filtered pooled urine each were taken and spiked with 100 µL of stock solution at 500–50 ng/mL or 50–5 ng/mL and 800 µL of sample diluent solvent, to have quality control (QC) urine samples at two levels of concentration.

Blind urines were obtained from the Swiss Laboratory for Doping Analyses and prepared according to the DS sample treatment procedure. Dilutions of either 10 or 100 times from original samples with ACN/H_2O 75:25 v/v were performed before injection (Table S2) to obtain comparable signal intensities among the samples.

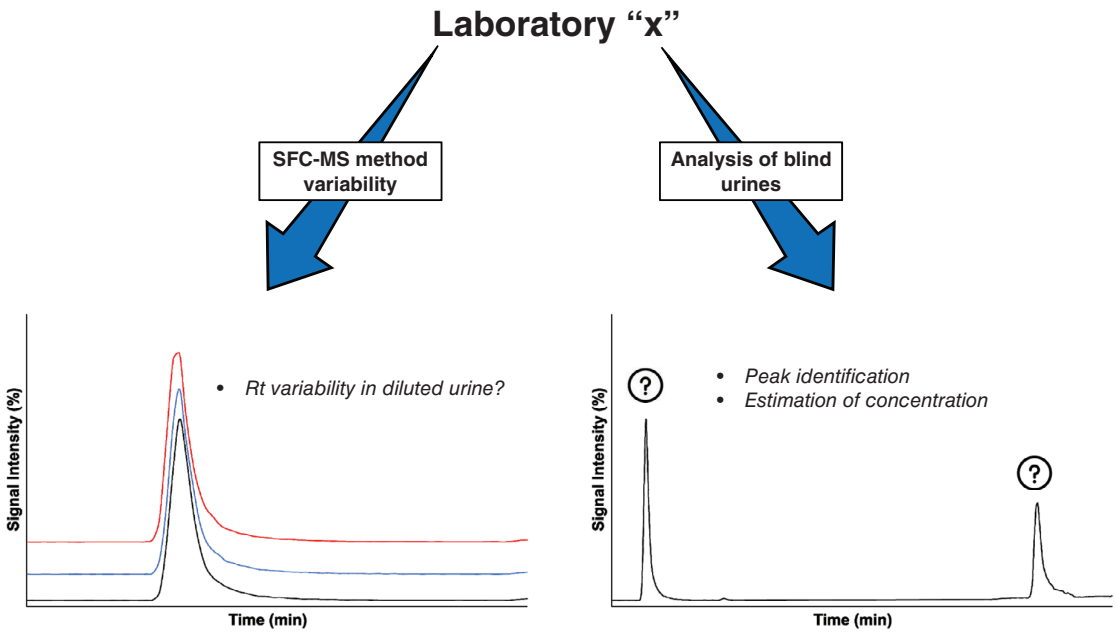

FIGURE 1 Description of the workflow employed in each laboratory participating in the study.

All samples have been prepared at the Swiss Laboratory for Doping Analyses and kept at a temperature of −22°C. Ready-to-inject vials containing negative quality control samples (blanks), positive control samples (QC), and blind urines were sent to all participating laboratories.

2.3 | UHPSFC-MS/MS instrumentation and chromatographic conditions

Each laboratory performed the analyses on a Waters Acquity UPC^2 system (Waters, Milford, MA, USA), equipped with a binary solvent delivery pump (BSM), an autosampler (SM), a column oven (CM), a UV detector (PDA) fitted with an 8 µL flow-cell and a two-step (active + passive) backpressure regulator (BPR). Each chromatographic system was hyphenated to a tandem mass spectrometer (triple quadrupole - QqQ; Table S3), equipped with an electrospray ionization (ESI) source. The hyphenation between the chromatographic and tandem MS systems was made via a dedicated double-T splitter interface from Waters. Additional make-up solvent (pure MeOH at a flow-rate of 0.3 mL/min) was brought by a Waters Isocratic Solvent Manager (ISM) pump.

Each MS/MS instrument operated in positive and negative ESI modes, using Selected Reaction Monitoring (SRM) as the acquisition mode. Polarity switching between the two ionization modes was performed within the same injection. Source temperature was set at 140°C. Nitrogen was used as the desolvation gas at 900 L/h and 450°C and as the cone gas at either 100 or 150 L/h. Argon was chosen as the collision gas. The capillary voltage was set at ± 1.0 kV. Individual values for transitions, cone voltages and collision energies, as well as the ionization mode have been listed in Table S4 of the Supplementary material. Dwell times were set at 20 ms for ESI positive, while a dwell time of 25 ms was chosen for the ESI negative.

The column employed by all laboratories was the Torus 2-PIC 100 × 3.0 mm ID 1.7 µm (Waters, Milford, MA, USA). The organic cosolvent was a mixture of MeOH/H_2O 98/2 v/v + 20 mM AmF. More information regarding the chromatographic method and the stationary phase choice can be found in.[8] Two Torus 2-PIC columns have been employed in this study. Data acquisition and instrument control were performed with MassLynx v4.1 or 4.2 (Waters, Milford, MA, USA), while data processing was performed with TargetLynx v4.1 (Waters, Milford, MA, USA).

2.4 | Sequence structure and data treatment

For the purpose of assessing the interlaboratory reproducibility, the sample sequence used by all laboratories was identical. Each laboratory was asked to perform a test of the UHPSFC-MS/MS method robustness using the QC samples (Figure 1). Subsequently, an identification and concentration estimation of potential doping agents in seven blind urines was carried out (Figure 1). A specific injection sequence was systematically applied in each laboratory: after multiple injections of a blank urine on the column, the two QC urine samples were injected in triplicate; then, the seven blind urines were also tested, with a double blank injection in between. Finally, the QC urine samples were once again injected in triplicate.

To assess the inter-laboratory retention times variability of the set of doping agents, the retention times obtained for all compounds present in each QC sample, injected at the beginning and end of

the sequence, have been used to perform the calculations. The total variance (s_T^2) was obtained using the following formula:

$$s_T^2 = s_r^2 + s_s^2 + s_l^2 \quad (1)$$

where s_r^2 represents the variance obtained between the injections of each triplicate analysis, s_s^2 represents the variability between the triplicate's injections at the beginning and end of the sequence and s_l^2 is the variance between laboratories. From the values of s_T^2 obtained for each compound, the relative standard deviation (RSD) was calculated to assess the inter-laboratory reproducibility. RSD values, represented in %, were plotted in a violin plot created using Plotly Chart Studio (https://chart-studio.plot.ly). Data treatment was performed via Microsoft Excel 2019.

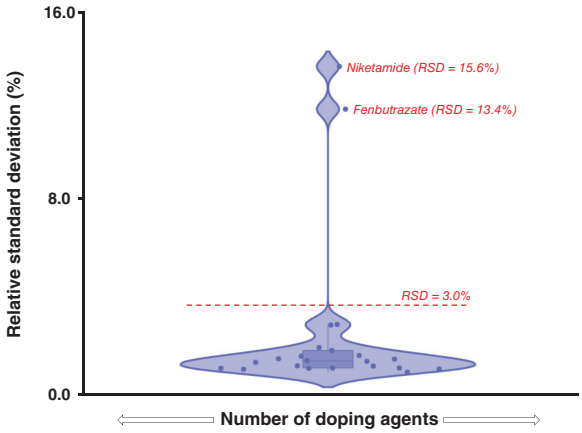

FIGURE 2 Violin plot representing the relative standard deviation (RSD%) values, relative to the inter-laboratory variability of retention times, obtained for the doping agents.

3 | RESULTS AND DISCUSSION

3.1 | Interlaboratory reproducibility

In a previous study revolving on the robustness assessment of a UHPSFC-MS/MS method for routine antidoping analyses, the combination of the latest generation UHPSFC stationary phases and the use of water as an additive in the mobile phase was successfully used to achieve an excellent stability of the analytical method.[8] The study, however, was carried out entirely in the same laboratory, raising still questions related to the potential inter-laboratory repeatability. This point must be properly assessed before UHPSFC can be considered as a viable alternative in routine laboratories. Only one work focusing on assessing the inter-laboratory reproducibility for a UHPSFC method was made, using a UV detector and simple pharmaceutical formulation.[15] In the present work, the robustness of a generic UHPSFC method using complex matrices (biological fluids), as well as an MS detector (MS/MS) hyphenated to the UHPSFC instrument was investigated.

This inter-laboratory evaluation of retention times variability was assessed for the 21 doping agents. Relative standard deviations (RSD), representing the variability of the retention times of each target compound found in each laboratory expressed in percentage, have been calculated and represented in a violin plot (Figure 2). Among the 21 analytes, 19 offer a suitable retention time repeatability between the four laboratories, with RSD (%) values below 3.0%. Only two compounds, namely niketamide and fenbutrazate, have shown a significant variability, as indicated by their position in the violin plot (Figure 2). Average RSD (%) values related to the intra-injection variability for these two analytes are also relatively high when compared to the other compounds (Table S5). To better investigate the reasons for this poor retention reproducibility, a correlation with the gradient conditions was made. In Figure 3A, the corresponding chromatograms of fenbutrazate, tamoxifen, and salbutamol were plotted. An immediate trend is visible from these three doping agents: a higher percentage of organic cosolvent in the mobile phase induces a better reproducibility of the retention times. The impact is further highlighted in Figure 3B: among the 21 doping agents, 19 of them elute quite late along with the chosen gradient profile (t_r > 2.7 min), while niketamide and fenbutrazate do not interact strongly with the stationary phase (no H-bond donor group on the structures) and are poorly retained. When these two compounds are eluted, the mobile phase is mostly under its supercritical state, due to the low percentage of cosolvent employed (between 2% and 4%). Under such conditions, the total backpressure of the system and mobile phase temperature can strongly impact retention.[17,18] This phenomenon, well-known in UHPSFC, is extremely hard to control and therefore, may have an important impact on the early-eluting compounds. In the present study, the small differences in the UHPSFC-MS system setup (ie, tubing dimensions, presence of switching valves, etc.; Table S3) and slight differences in pressure between 2-PIC columns, generate some differences in total system pressure between laboratories and could explain the variability of early-eluting compounds. Besides the pressure differences between the UHPSFC systems used in different laboratories, the system extracolumn volume might also cause further variability in the retention profile, especially for early-eluting compounds. The UHPSFC system employed by all laboratories (Waters Acquity UPC^2) has already been characterized as an instrument with an important extracolumn volume.[19] This additional volume translates into an increase of the retention times, especially for early-eluting compounds. The changes in the retention times highly depend on the system setup, therefore even minimal changes in the extracolumn volume of the UHPSFC system can translate into an important variability for those analytes who do not interact well with the stationary phase. Due to the low retention times for niketamide and fenbutrazate, a variation of their retention can have a higher impact on the calculation of their variability compared to those analytes with high retention times. Moreover, the two Torus 2-PIC columns employed do not belong to the same batch, which might potentially have also contributed to the variability seen for these two analytes among the four laboratories. Finally, from Figure 3A, it is possible to also see a peak-splitting phenomenon for fenbutrazate, another common issue with early-eluting compounds.

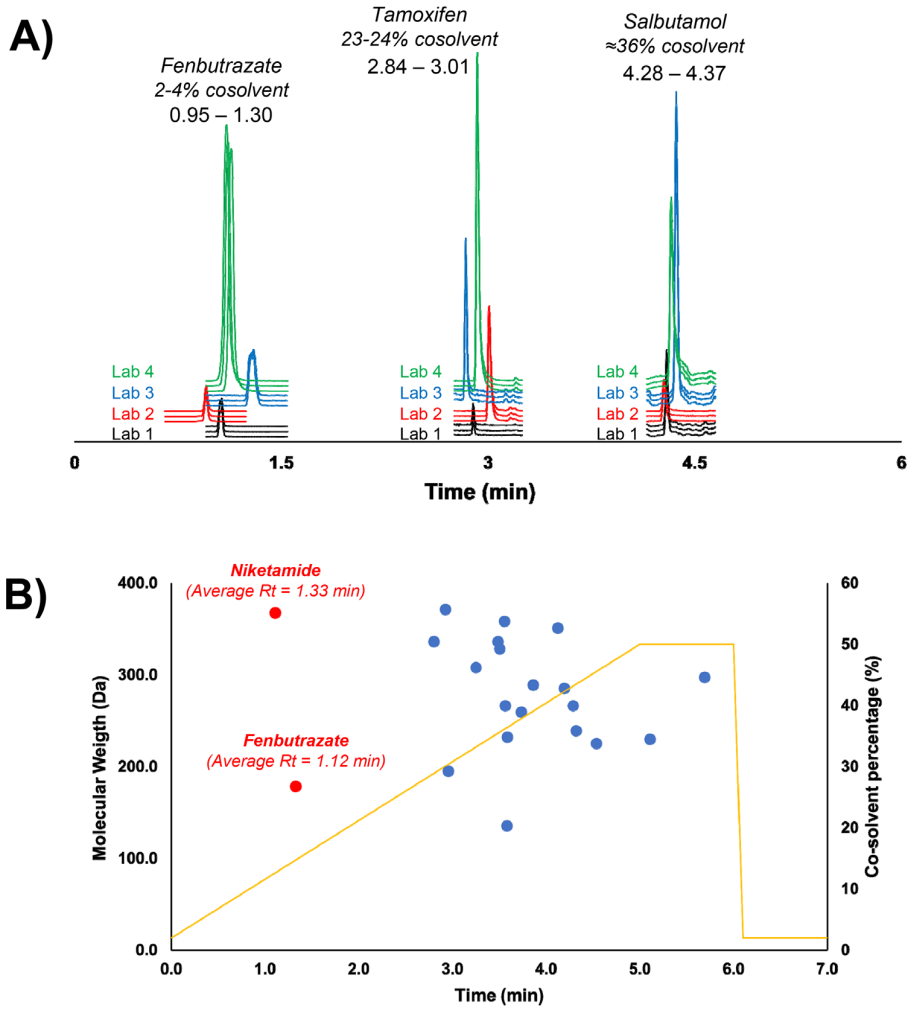

FIGURE 3 A) Overlay of the chromatograms for three compounds (fenbutrazate, tamoxifen, and salbutamol) obtained by each laboratory; B) Plot of the average retention times, obtained by the four laboratories, for the 21 doping agents across the gradient profile used in this study.

Regardless of the unsatisfactory results found for niketamide and fenbutrazate, the variability of retention times was low for the majority of compounds when analyzed in the four laboratories. The employment of important percentages of the liquid organic modifier contributed to the overall method robustness. More importantly, as already mentioned in,[8] the use of water as a mobile phase additive, together with the use of the latest generation of UHPSFC stationary phase (Torus 2-PIC), cause a substantial improvement in retention reproducibility under UHPSFC conditions. While in[8] this statement was verified only in one laboratory, this work confirms that such method robustness was maintained between different laboratories.

3.2 | Analysis of blind urine samples

Following the evaluation of the inter-laboratory reproducibility, the performance of the developed UHPSFC-MS/MS method was assessed, by analyzing a set of seven different urine samples, each containing zero to two of the previously discussed 21 target compounds. These excretion urine samples were called "blind urines" and were used to demonstrate the fitness of the method for routine anti-doping analysis purposes. Each laboratory performed the analysis without knowing which, and how many analytes were present in each blind urine, nor their concentration. The aim was, quite simply, to verify how all laboratories were capable to properly perform a routine screening for urine samples, consisting of the identification step, as well as a rather simple estimation of the concentration of the doping agents. In Figure 4, the chromatograms of the different compounds present in six blind urines are represented. Blind urine 7 did not contain any doping agent, while two doping agents (amiloride and hydrochlorothiazide) were present in blind urine 4. First, all doping agents were successfully identified by all laboratories; the window range in retention time between the four laboratories was kept to a minimum, with five of the six compounds eluting within a window of 0.12 min on the maximum (Figure 4A). Hydrochlorothiazide, present in blind urines 4 and 6 (Figure 4B,C), is the only sample presenting extended elution window (0.34 min), due

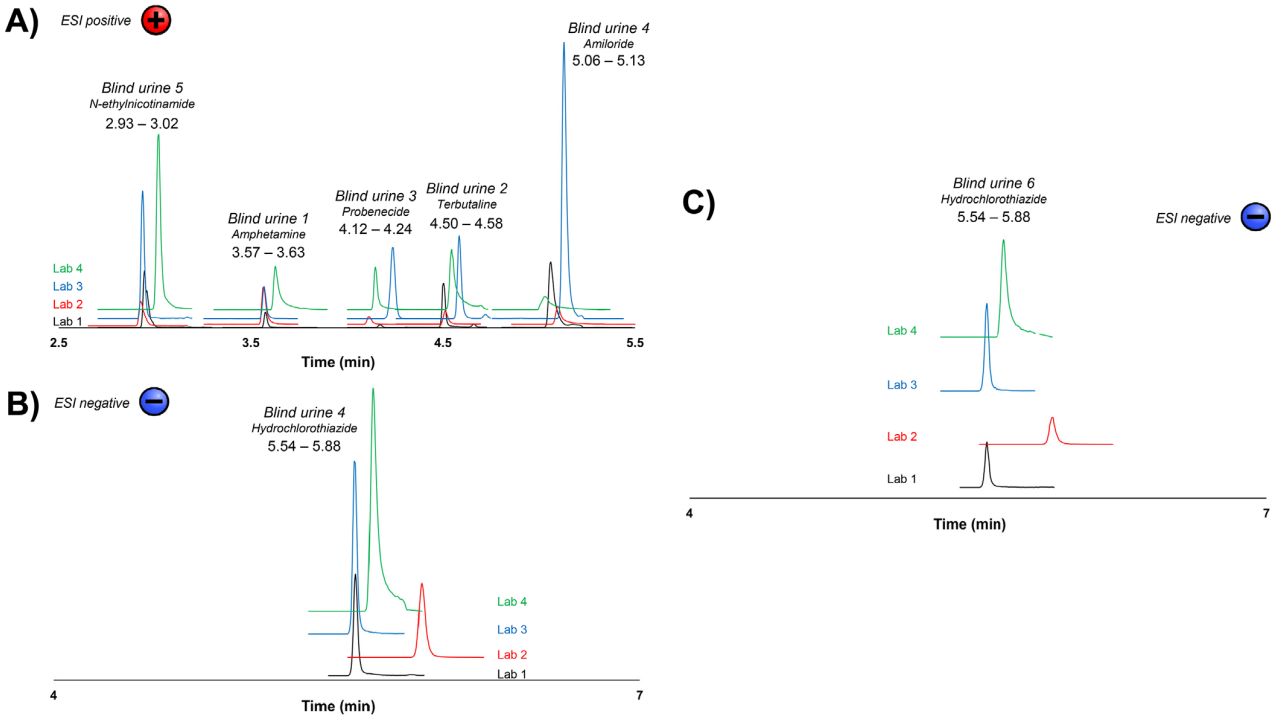

FIGURE 4 A) Chromatograms of each analyte (N-ethylnicotinamide, amphetamine, probenecide, terbutaline, and amiloride) present in blind urine samples 1 to 5 using ESI positive mode by all four laboratories; B) Chromatograms of hydrochlorothiazide found in blind urine 4 using ESI negative mode, obtained by all four laboratories; C) Chromatograms of hydrochlorothiazide found in blind urine 6 using ESI negative mode, obtained by all four laboratories

TABLE 1 Estimated concentration values of all found analytes in blind urine samples 1 to 6 from all four laboratories, compared against their related MRPL levels

Estimated concentration (ng/mL)						
	Blind urine 1 (Amphetamine)	Blind urine 2 (Terbutaline)	Blind urine 3 (Probenecide)	Blind urine 4 (Amiloride + Hydrochlorothiazide)	Blind urine 5 (Niketamide metabolite)	Blind urine 6 (Hydrochlorothiazide)
Laboratory 1	89	8	408	26246	22	54
Laboratory 2	69	8	463	39481	24	77
Laboratory 3	81	10	461	36323	21	71
Laboratory 4	109	8	408	36796	25	151
MRPL	*100*	*20*	*200*	*200*	*100*	*200*

to the shift in the retention profile witnessed by laboratory 2, although the calculated RSD was only equal to 2.8%.

Having assessed the identification step, the focus shifted toward the estimation of the concentration for each analyte. By using the same doping agents spiked in the quality control samples at two different concentration levels, a two-point calibration curve for each identified analyte was made. Due to the limited number of QC samples, only a simple estimation of each unknown analyte was possible. The curves were, then, used to estimate the concentrations of each doping agent found in the seven blind urines. The estimated concentrations, described in Table 1, have been compared to the respective Minimum Required Performance Levels (MRPL) values for each doping agent. No values from blind urine 7 have been shown, as no substances were present in this sample. The results found by the four laboratories were overall consistent for all analytes present in the six blind urines (Table 1), illustrating the potential of UHPSFC-MS/MS during the screening process in anti-doping analyses. Although some differences, regarding the estimated concentrations, were seen (Table 1), it should be noted that the MS/MS systems used by the four laboratories were not identical, although they consisted of the same MS analyzer type (triple quadrupole). This difference in the UHPSFC-MS/MS configuration could explain the differences in the estimations, especially

if a possible saturation of the signal intensity occurred. Regardless of the differences observed between some laboratories, the UHPSFC-MS/MS method was capable of giving the same results, when considering the relative MRPL values.

4 | CONCLUSIONS

In this work, an assessment of the interlaboratory reproducibility of a UHPSFC-MS/MS method between four laboratories has been made on a set of 21 doping agents spiked in a biological matrix. The results showed acceptable robustness of the method, with a low variability of the retention times for 19 out of 21 analytes. This was associated with the employment of an important amount of organic cosolvent in the mobile phase. For two early-eluting compounds, nikethamide and fenbutrazate, the observed variabilities were higher, indicating that in future method development and inclusion of new compounds in the method, there might be potential issues in terms of retention times reproducibility for those compounds that elute with a limited amount of organic cosolvent in the mobile phase. Moreover, the influence of the instrument volume should not be neglected for such target analytes, as it might negatively impact the reproducibility of their retention times, too.

In the second part of this article, the analysis of a set of seven blind urine samples was performed. Each laboratory has successfully identified the unknown analytes present in blind urines. Moreover, the estimation of the concentrations for each unknown doping agents performed across the four laboratories gave consistent results overall, when compared to the respective MRPL values. These findings indicate, therefore, that UHPSFC-MS/MS has managed, in these years, to evolve into a technique which could be potentially employed for screening procedure in antidoping laboratories.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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II.3 Theoretical considerations when implementing high percentages of organic modifier in UHPSFC

II.3.1 Use of unconventional temperatures in UHPSFC

In the past 5-7 years, there have been different attempts in expanding the application range of UHPSFC. The main limit was represented by the poor elution power of the mobile phase for analytes with an elevated polarity, as well as by their poor solubility under the typical UHPSFC mobile phase. To overcome these issues, researchers have begun implementing a new gradient profile, called “Unified Chromatography” (UC). With UC, the analysis would start with the usual $scCO_2$ to modifier ratio (98:2 or 95:5 *v/v*), but instead of limiting the co-solvent percentage to 40-50%, it would allow a higher presence of the liquid component, reaching up to 100% of modifier in some cases. The idea behind this new concept is simply to increase the amount of the polar organic solvent in the mobile phase as much as possible, enabling the elution of the most retained analytes as well as limiting the precipitation issues observed with the more polar and hydrophilic substances.

UC gradients are fairly new in the context of UHPSFC analyses, and there is a need to investigate how different factors may impact the overall quality of the separation. Indeed, with a gradient ramp starting at 2% of co-solvent and reaching percentages as high as 100%, several state transitions of the mobile phase should be expected. Initially, a supercritical fluid is present, but as the co-solvent increases, a gradual shift towards a liquid-like fluid should be obtained, reaching a complete liquid state of the mobile phase at the end of the analysis. This can greatly impact the mobile phase density, its linear velocity (at a fixed flow-rate) and the kinetic performance generated, as well as the retention profile and the quality of the separation. Hence, a re-evaluation of the impact of some factors needs to be performed. Among those, column temperature is a highly relevant parameter, especially with UHPSFC. Changes in temperature are known to generate variations in the mobile phase density: with higher temperatures the density decreases, thus increasing the compound’s retention. This is true with a super/subcritical mobile phase, at modifier percentages limited to 30-40%, however a change in the pattern has been already witnessed when the modifier’s presence increases, with a LC-like behavior starting to appear with temperature changes (e.g. decrease in the retention with higher temperatures).

In this third article an overview of how an extended column temperature range (from 80°C to -5°C) can impact UHPSFC analysis is given. Kinetic and thermodynamic considerations have been discussed, by performing analyses at four different mobile phase’s ratios. Finally, some applications demonstrating the usefulness of unusual temperatures in UHPSFC are presented.

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Investigating the use of unconventional temperatures in supercritical fluid chromatography

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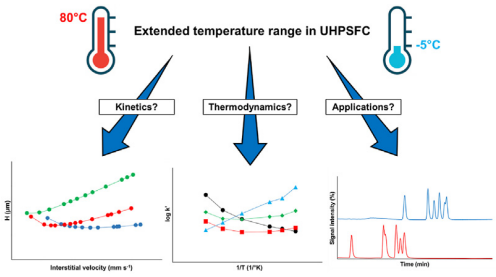
HIGHLIGHTS

- Interesting kinetic performance recorded at high or low temperatures at different cosolvent percentages.
- Up to 3 different chromatographic retention patters identified with temperature variations.
- Excellent stability of the Torus 2-PIC stationary phase at high temperatures.
- The use of high and low temperatures seem to be sometimes beneficial in the method development process.

GRAPHICAL ABSTRACT



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ABSTRACT

The use of unorthodox temperatures, ranging from −5 °C up to 80 °C, have been thoroughly investigated in supercritical fluid chromatography. To this purpose, an initial evaluation of the kinetic and thermodynamic performance has been made with a set of 4 analytes eluting at different percentages of organic co-solvent in the mobile phase (3%–10% - 45%–80%). The van Deemter plots have demonstrated how, at low organic modifier presence, the use of low temperatures did not necessarily translate into worse performance, while high temperatures could pose more issues due to the poor handling of the super/subcritical mobile phase by the chromatographic system. With important percentages of co-solvent, however, high temperatures were fundamental in ensuring better profiles of the van Deemter plots, compared to low temperatures. Pressure plots have demonstrated that gradients reaching elevated percentages of organic modifiers can also be used on stationary phases packed with sub 2 μm silica particles if high temperatures are employed. The thermodynamic evaluation, made via the analysis of van't Hoff plots, indicates the presence of three retention behaviors happening in UHPSFC when switching from high to low temperatures, depending on the co-solvent percentage needed to elute one analyte. Finally, an assessment of the stationary phase stability at high temperatures was performed: the retention times variabilities recorded were minimal (RSD < 2.5%), as well as the peak widths and inlet column pressures were somewhat constant throughout the analyses.

In the second part of this study, a focus on potential applications benefiting from such unconventional temperatures has been made. A series of challenging analytes have experienced better chromatographic resolution at either high or low temperatures, providing therefore a potentially interesting tool to analysts during the chromatographic method development process. In conclusion, the UV sensitivity at

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different temperatures was also taken into consideration, with no significant impact on the quality of the UV signal under any condition.

1. Introduction

The development of an efficient chromatographic method remains challenging, as several parameters may present an impact on the separation profile. In liquid chromatography (LC), the stationary phase chemistry and mobile phase composition (organic solvent nature and proportion, additives, etc.) are the most important factors taken into consideration during the method development process [1,2]. In addition, there are other variables that are considered of secondary importance, thus possessing a lower impact on the separation profile [3,4]. Among these, column temperature is certainly the most important one to further improve the chromatographic separation. In LC, the role of column temperature has been extensively studied [5–9]. The use of elevated temperature is generally associated with a decrease of mobile phase viscosity and polarity (e.g. decrease of water polarity). The lower viscosity results in a significant decrease of column pressure drop and an increase in the analytes' diffusion, thus shifting the optimal mobile phase velocity to higher values, therefore enabling to speed up the separation without suffering from high backpressures. The polarity reduction of water allows decreasing the amount of organic modifier in the mobile phase at elevated temperature, while maintaining comparable retention in reversed phase mode LC. On the other hand, the use of low temperature has been found to be beneficial for modifying selectivity in LC, in particular for closely related compounds such as positional isomers, diastereomers and even enantiomers [9].

In ultra-high performance supercritical fluid chromatography (UHPSFC), the situation is quite different. First of all, the method development strategy relies almost exclusively on the stationary phase screening [10–12] and, to a lower degree, on the mobile phase optimization [13,14]. Other parameters are relegated to a secondary role: the backpressure set by the backpressure regulator module (BPR), for example, was used in the past to modulate the retention of some compounds [15–17], but it has nowadays lost most of its interest. Similarly, column temperature has been scarcely optimized in recent UHPSFC applications [17,18]. However, both parameters are relevant, due to the nature of the UHPSFC mobile phase, characterized by a much higher compressibility compared to that of liquids. . Consequently, an increase of the mobile phase temperature can be often associated to a reduction of the mobile phase density and vice versa [19,20].

In the last few years, a significant number of UHPSFC methods were developed with a large amount of co-solvent in the mobile phase, equal to or greater than 40–50% (Fig. 1) [21–24]. In addition, water (up to 5–7% in the co-solvent) as well as salts or buffers (≥20 mM in the co-solvent) are commonly used as mobile phase components [23–25]. The implementation of these changes in the UHPSFC mobile phase might result in different behaviors when an extended temperature range, with a potential impact also on the mobile phase viscosity and density. Thus, former knowledge on the role of column temperature needs to be revisited.

The aim of this work was to study the impact of column temperature in UHPSFC conditions. A wide range of temperatures was tested, ranging from 80 °C down to a *sub-zero* environment (−5 °C). The theoretical aspects have been assessed under the above-mentioned conditions. First, a kinetic evaluation was performed, via the systematic investigation of plate height curves. An assessment of the pressure drops recorded was also made, since UHPSFC systems have limited upper pressure range (max 40–66 MPa). Van't Hoff plots were also constructed to assess the impact of a wide temperature range on the retention of a set of four representative compounds, at different co-solvent percentages in the mobile phase. The practical benefits of using a wide temperature range in UHPSFC were also demonstrated. This includes an assessment of the impact of high temperatures on the performance of the UHPSFC stationary phase over a prolonged timeframe; an evaluation of the impact of very high or low temperatures on the selectivity of different sets of closely-related pharmaceutical compounds and an investigation of the signal-to-noise ratio, at different temperatures with UV detector, using a mixture of a commercially available pharmaceutical API and its main impurities.

2. Materials and methods

2.1. Chemicals, reagents and sample preparation procedures

Methanol (MeOH), ethanol (EtOH) and acetonitrile (ACN) of OPTIMA LC/MS grade and water of UHPLC grade were purchased from Fisher Scientific (Loughborough, UK). Pressurized carbon dioxide (CO_2) 4.5 grade (99.995%) was purchased from PanGas (Dagmerstellen, Switzerland). Butylparaben, uracil, maleic acid, indoxyl sulphate, caffeine, adenosine, dexamethasone, betamethasone, (E/Z)-endoxifen, ammonia solution at 25% *v/v* of MS-grade and ammonium formate were all purchased from Sigma-Aldrich (Buchs, Switzerland). Paroxetine hydrochloride anhydrous, paroxetine impurities A, C and H were all purchased as European Pharmacopoeia (EP) reference substances from EDQM (Strasbourg, France). 2β-/19-/6α-/15β-/11β-/2α-/16α-hydroxytestosterone samples were all purchased from Steraloids (Newport, RI, USA).

Different sample preparation procedures have been applied for all analytes. All details related to sample preparation can be found in the first section of the supplementary material.

2.2. UHPSFC-UV-MS instrumentation, columns and chromatographic conditions

All experiments were performed on a Waters Acquity UPC^2 system (Waters, Milford, MA, USA) equipped with a Binary Solvent Manager delivery pump, a Sample Manager autosampler which included a 10 μL loop for partial loop injection, a column oven with active preheater, a PDA detector and a two-step (active and passive) backpressure regulator (BPR). Such configuration was employed for analyses in the column temperature range from 80 °C down to 5 °C. To perform the analysis at −5 °C, an external device was implemented on the chromatographic system (Fig. S3). The setup includes an inlet stainless steel passive preheater with a geometry of 800 × 0.125 mm I.D. and an outlet stainless steel tubing from the column outlet to the UV detector of 750 × 0.175 mm I.D. The cooling procedure to −5 °C consisted in inserting the column into a watertight cylinder made of stainless steel and branching such container to a chiller pump which delivered a mixture of polyethylene glycol/water 40/60 *v/v* cooled down to a temperature of approximately −7 °C into the cylindric container. The column

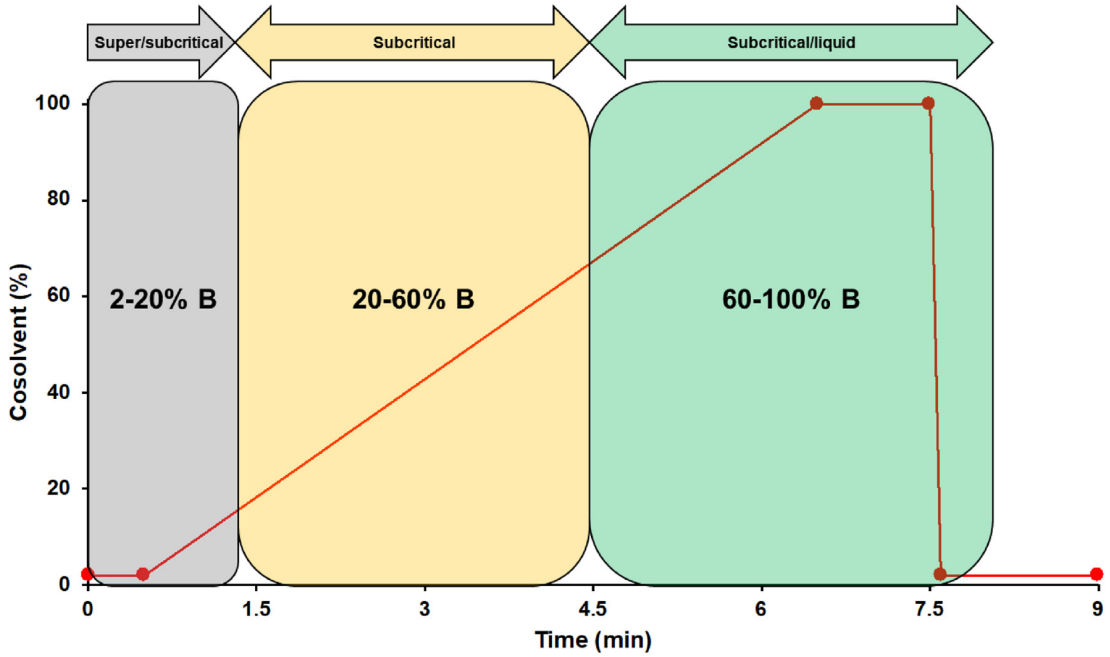

Fig. 1. Illustration of a generic Unified Chromatography (UC) gradient profile, with the consequent changes in the mobile phase state.

temperature was directly measured inside the container. Pure ACN was used as the weak wash solvent, while a mixture of MeOH/H_2O 50/50 was employed as the strong wash solvent, with volumes of 600 and 200 μL respectively. The autosampler module was constantly kept at a temperature of 8 °C. The injection volume was fixed at 1.5 μL for all analyses. The automated back-pressure regulator module (ABPR) was kept at a fixed value of 12 MPa for all analyses (constant outlet pressure). For the fundamental part of the work (construction of plate height curves and van't Hoff plots), UV detection was employed at different wavelengths (butylparaben at 255 nm; uracil at 249 nm; maleic acid at 220 nm and indoxyl sulphate at 237 nm).

In the second part of this work (paragraph 3.5), for the analysis of selected compounds at high and low temperatures, a single quadrupole mass spectrometer (Waters Acquity QDa) was also coupled to the UHPSFC system, with the addition of a Waters Acquity Isocratic Solvent Manager (ISM) make-up pump, via the employment of a "*pre-BPR splitter with make-up pump*" SFC-MS interface. A detailed description of such interface can be found elsewhere [26]. The make-up pump delivered pure MeOH at a fixed flow-rate of 0.15 mL min^{-1}. For MS detection, capillary voltage and cone voltage were equal to 1.5 kV and 25 V, respectively. Source temperature was fixed at 450 °C.

Data acquisition, data handling and instrument control were all performed via Empower 3.0 software (Waters). Data treatment was performed using Microsoft Excel 2019 and Tibco Statistica.

A set of five columns, all packed with Torus 2-picolylamine (2-PIC) 1.7 μm stationary phase in different column geometries, have been used in this study, and were generous gift from Waters. One 50 × 3.0 mm I.D. Torus 2-PIC was used to collect data for the plate height curves. For the remaining experiments, a set of four 100 × 3.0 mm I.D. Torus 2-PIC columns have been employed. Different chromatographic conditions have been applied throughout the work. All the chromatographic details were included in section 2 of the supplementary material.

2.3. Plate height curves construction

Plate height curves are widely used to study the kinetic performance in chromatography. Several plate height equations have been developed in the past, and among them, the one proposed by van Deemter is still the most frequently used to determine the optimal mobile phase velocity and the minimum achievable plate height. Its simplified form (Eq. (1)) can be written as:

$$H = A + \frac{B}{u} + C^{*}u \quad (1)$$

where H corresponds to the plate height, u is the mobile phase linear velocity, while A, B and C are the coefficients of the Van Deemter equation. In the present study, van Deemter plots were constructed for different co-solvent percentages in CO_2 (i.e. 2-5%, 10–15%, 45–50%, 75–100%) at five temperatures (80 °C, 40 °C, 15 °C, 5°Cand -5°C) under isocratic conditions. Four analytes have been injected in selected mobile phase compositions (corresponding to different co-solvent percentages in CO_2), to maintain comparable solute retention. The compounds were: butylparaben for the 2–5% range of organic modifier, uracil for the 10–15%, maleic acid for the 45–50% and, indoxyl sulphate for the 75–100%. The plate numbers (N) were calculated via Empower according to the European Pharmacopoeia definition. From the plate number values, the plate heights (H) were derived and used to construct the plate height curves. Interstitial velocities, which correspond to the mobile phase linear velocity, were determined by injecting butylparaben.

In order to take into consideration the extra-column system dispersion, measurements were performed by replacing the column with a zero dead volume (ZDV) union connector and injecting butylparaben as model analyte to estimate extra-column band broadening (variance). Injections were performed under the same conditions as those made with columns, at all the flow-rates, temperatures and co-solvent proportions previously tested. Plate heights were then corrected for extra-column peak dispersion. The

extra-column volumes of the two system configurations (original or external oven) were measured under LC conditions, using 100% MeOH as mobile phase, to avoid problems related to the mobile phase compression. The residence time of butylparaben was measured at various flow rates. The system volume was determined from the relationship between residence time and reciprocal flow rate. More information can be found in the Supplementary material.

Viscosity estimation of CO_2 was based on an empirical correlation proposed by Ouyang [27]. Then, viscosity was predicted for different CO_2 and MeOH mixtures using a correlation of second order between viscosity and MeOH content, on the basis of experimentally determined data published by Sih*et al.* [28]. Finally, combining these correlations allow the prediction of viscosities for any mixtures of supercritical CO_2 and MeOH at a given pressure and temperature [29].

2.4. Van't hoff plots construction

Van't Hoff plots are widely used to model the retention profile of a probe compound under specific analytical conditions (thermodynamic behavior). To do so, the previously selected analytes (i.e., butylparaben, uracil, maleic acid and indoxyl sulphate) have been injected first in isocratic mode at four co-solvent levels in the mobile phase: 3% of co-solvent for butylparaben, 15% for uracil, 45% for maleic acid and 80% for indoxyl sulphate. The logarithms of retention factors have been plotted, against reciprocal temperature, according to the common form of Van't Hoff equation (Eq. (2)):

$$\ln k = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} \quad (2)$$

where ΔH° is the enthalpy exchange, ΔS° is the entropy exchange, R is the universal gas constant and T is the column temperature.

As a second step, Van't Hoff plots were also constructed under isopycnic conditions. To do so, the column inlet pressure was maintained at 30 MPa – whatever the temperature and mobile phase composition - by adjusting the BPR.

3. Results and discussion

3.1. Theoretical aspects: kinetic evaluation

3.1.1. Plate height curves under supercritical conditions

The implementation of a gradient ranging from 0 to 100% of organic modifier, at column temperatures normally employed in UHPSFC applications, namely in the range between 25 °C and 50 °C, is expected to affect the nature of the mobile phase, as illustrated in Fig. 1. The increasing addition of a liquid cosolvent causes the shift from the initial supercritical conditions to subcritical ones, possibly even reaching a fully liquid state in some cases. Using such diverse conditions in UHPSFC generates important variations of the mobile phase properties, requiring therefore an update on the knowledge of the impact of column temperature in UHPSFC. To revisit this aspect, the kinetic performance under different temperature conditions (from 80 °C to *minus* 5 °C) have been initially evaluated. It is of prime importance to understand at which column temperature the best performance can be reached, considering the different mobile phase compositions that are starting to become, nowadays, more common in UHPSFC. All experiments have been performed on a Torus 2-PIC stationary phase; this choice was mainly motivated to the presence of hybrid silica particles, which possess a better resistance to high temperatures compared to normal silica. Therefore, van Deemter (VD) curves have been plotted at five different column temperatures for a set of four analytes eluted under the different mobile phase conditions depicted in Fig. 1. For each analyte, an attempt in keeping the retention factor (k) to a minimal value of at least 5 was made, with the purpose of minimizing the influence of extra-column band broadening on the chromatographic peaks. Moreover, to ensure the validity of H values, the latter were systematically corrected for the extra-column peak dispersion. In Fig. 2a, the VD plots are reported for butylparaben. As shown, the change in column temperature has an important impact on the minima (H_{min}) of the VD plots as reported in Table 1 (H_{min} values comprised between 3.5 and 6.1 μm). In LC, the use of different temperatures does not impact H_{min} values, but only the optimal velocities. In UHPSFC, however, variations of H_{min} can be observed (Fig. 2a). In Fig. S4 of the supplementary material, the retention factors obtained for butylparaben at the five different column temperatures have been plotted against the mobile phase linear velocity. Under LC conditions, the retention factor of the analyte experiences none, or very little, change at a fixed temperature when varying the linear velocity. This notion becomes very important when performing van Deemter plots, as the B and C terms of the van Deemter equation are directly linked to the retention factor. In UHPSFC, on the other hand, retention factors change when moving from low to high velocities, at a fixed column temperature (Fig. S4). Moreover, such k variation becomes quite impressive with the increase of the column temperature and limited cosolvent percentage, as witnessed at 80 °C for butylparaben (Fig. S4a). Analyses at 80 °C have been, therefore, remade by regulating the ABPR value in order to reach a total column inlet pressure of approximately 30 MPa. With these "isobaric" conditions the shift in the k values, at 80 °C, from high to low velocities has been greatly reduced, simulating the one observable at the other temperatures (Fig. S4e). Nonetheless, the VD plot obtained in "isobaric" conditions greatly resembles the "non-isobaric" one, indicating, more importantly, that it was not possible to reach the H_{min} due to an instrumental limitation of the UHPSFC system used in this study, as the upper flow rate limit is fixed at 4 mL min^{-1}. This instrumental constraint decreases the interest of working at high temperature (80 °C) in presence of a limited amount of co-solvent in the mobile phase, as it poses a serious constraint in properly assessing the VD plot minimum.

On the other hand, the shift from 40 °C to lower temperatures has also produced an interesting pattern. The lowering of the column temperature from 40 °C to −5 °C translated into a small impact on the difference between the H_{min} values between those two points. Moreover, the minimum reduced plate height (h_{min}) reached a value close to 2 in *sub-zero* conditions. The reduction of column temperature below 0 °C has therefore generated only a minimal loss in efficiency compared to 40 °C. This might be explained by a compromise between, on one hand, the negative effect of low temperature on the *C*-term (related to mass transfer resistance) visible in Fig. 2a and, on the other hand, the potential benefit of having a more homogeneous mobile phase (at −5 °C, the supercritical carbon dioxide can be found, in a *sub-zero* environment, in a more liquid-like state which would translate into an increase of its density).

3.1.2. Plate height curves under subcritical conditions

Two additional mobile phases containing higher proportions of co-solvent were tested, namely 10–15% and 45–50%. Here, the mobile phase cannot be considered as a supercritical fluid [30]. It was demonstrated that, instead, "subcritical" was a more precise term [30]. Fig. 2b shows the VD plots obtained with uracil, analyzed with a mobile phase composed of 90/10 CO_2/modifier *v/v*. In this case, the curves present an excellent overlap at 40, 15 and 5 °C. At −5 °C, a very similar H_{min} was also obtained, although at lower velocities. This translates into the potential use of any kind of

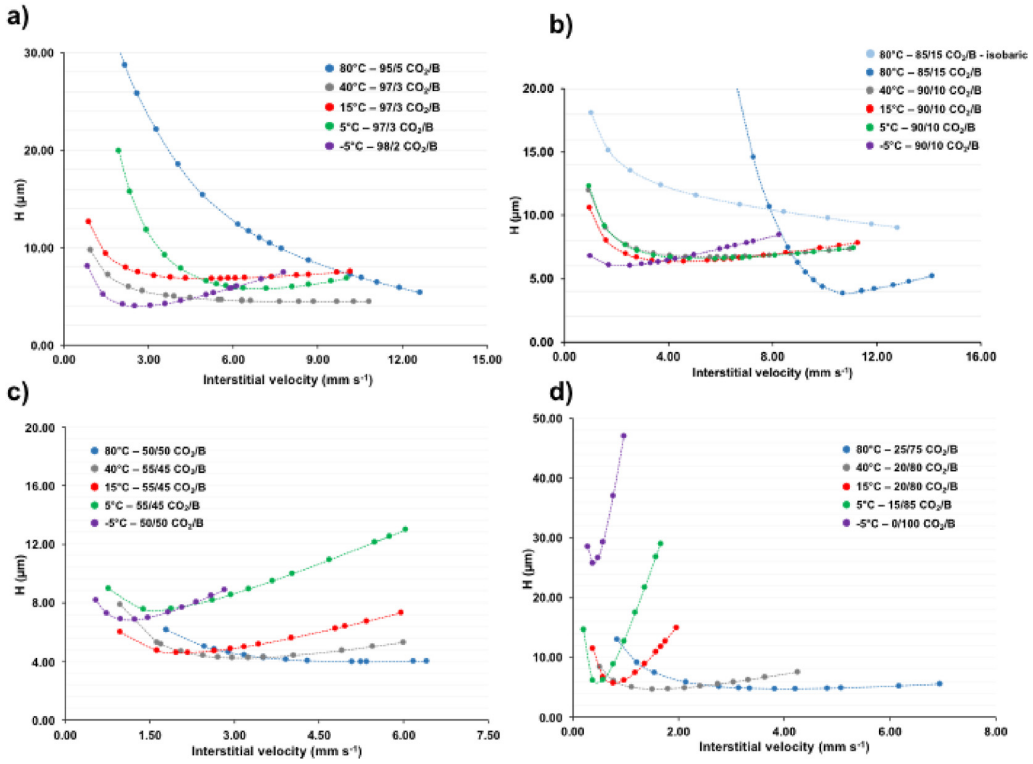

Fig. 2. a) van Deemter plots, corrected by the instrument volume, obtained for butylparaben at different cosolvent percentages and at five temperatures: 5% - 80 °C (blue), 3% - 40 °C (grey), 3% - 15 °C (red), 3% - 5 °C (green) and 2% to −5 °C (violet). Cosolvent used: MeOH/H_2O 95/5 *v/v*. b) van Deemter plots, corrected by the instrument volume, obtained for uracil at different cosolvent percentages and at five temperatures: 15% - 80 °C (blue), 15% - 80 °C isobaric (light blue), 10% - 40 °C (grey), 10% - 15 °C (red), 10% - 5 °C (green) and 10% to −5 °C (violet). Cosolvent used: MeOH/H_2O 95/5 *v/v*. c) van Deemter plots, corrected by the instrument volume, obtained for maleic acid at different cosolvent percentages and at five temperatures: 45% - 80 °C (blue), 50% - 40 °C (grey), 50% - 15 °C (red), 50% - 5 °C (green) and 45% to −5 °C (violet). Cosolvent used: MeOH/H_2O 95/5 *v/v* + 10 mM of ammonium formate. d) van Deemter plots, corrected by the instrument volume, obtained for indoxyl sulphate at different cosolvent percentages and at five temperatures: 45% - 80 °C (blue), 50% - 40 °C (grey), 50% - 15 °C (red), 50% - 5 °C (green) and 45% to −5 °C (violet). Cosolvent used: MeOH/H_2O 95/5 *v/v* + 10 mM of ammonium formate. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Optimal velocities, H_{min}, optimal flow-rates and reduced h_{min} values obtained at the different tested conditions.

T (°C)	Analyte	u_{opt} (mm/s)	F_{opt} (mL/min)	H_{min} (µm)
80	Butylparaben	12.6	3.8	5.8
	Uracil	9.6	2.1	5.1
	Maleic acid	6.4	2.2	3.8
	Indoxyl sulphate	3.3	0.8	4.5
40	Butylparaben	6.6	2.2	3.5
	Uracil	6.2	2	6.4
	Maleic acid	3.2	1.3	3.9
	Indoxyl sulphate	1.5	0.5	4.6
15	Butylparaben	5.2	1.8	6.1
	Uracil	4.5	1.5	6.1
	Maleic acid	1.6	0.5	4.3
	Indoxyl sulphate	0.7	0.4	5.9
5	Butylparaben	5.4	1.9	5.2
	Uracil	4.7	1.5	6
	Maleic acid	1.3	0.5	5.7
	Indoxyl sulphate	0.3	0.2	6.2
−5	Butylparaben	2.5	0.9	3.8
	Uracil	1.7	0.5	5.8
	Maleic acid	0.7	0.3	6.9
	Indoxyl sulphate	0.3	0.1	24.6

column temperature in such range without suffering from severe loss of kinetic performance. On the other hand, the plate height curve obtained at 80 °C has a completely different shape, with a severe decrease of efficiency at low velocities (Fig. 2b). The van Deemter graph was replotted under isobaric conditions, in a similar way as explained in the previous paragraph (Fig. 2b). With a constant column inlet pressure at 30 MPa, the VD plot has changed its shape, indicating that once more with extremely high column temperatures, such as 80 °C, it is not possible to reach the minimum of the van Deemter plot due to instrumental limitation, similarly to what it has been described for butylparaben under supercritical conditions. At lower temperatures, however, no change in the VD plots has been observed.

In Fig. 2c, the van Deemter plots for maleic acid are reported. For this compound, a higher percentage of co-solvent was required (around 45–50%). With these conditions, the mobile phase is transitioning to a more liquid state. Therefore, such change should correspond to a shift towards UHPLC-like behaviors and the VD plots in Fig. 2c support this claim. The highest kinetic performance was obtained at high temperature, meaning lowest H_{min} values at higher velocities (u_{opt}) (Table 1). Moreover, the VD curve at 80 °C depicts a good flattening after the u_{opt} value, which is another phenomenon also observed under UHPLC at elevated temperatures thanks to a decreased *C* term contribution to the overall plate height. All these proofs indicate that, with such ratio of CO_2 to organic modifier, UHPLC-like behavior should be expected at high temperatures. On the other hand, low column temperatures have been associated to an increase in the *C* term value (Fig. 2c), as normally seen in UHPLC [31]. In addition, when lowering the temperature down to 5 °C and −5 °C, H_{min} values strongly

increased in comparison to those observed at 15, 40 and 80 °C (Table 1).

3.1.3. Plate height curves under liquid-like conditions

A recent trend that has regained interest in UHPSFC consists in using very high organic modifier percentages, up to 100% in the mobile phase. The use of such important amounts of liquid cosolvent, which enable analysts to reach LC conditions, was already the subject of previous works [23,24,32,33]. The rationale behind this choice is based on the possible expansion of the application range of UHPSFC towards highly polar molecules. A few promising applications have already been developed deriving from the benefit of employing limited percentages of CO_2 in either pure methanol or in a methanol-water mixture. It has become quite evident, therefore, that new applications might arise in the future using such conditions. To help the method development process, the impact of column temperature was assessed also under such unconventional conditions. Indoxyl sulphate was chosen as a test compound and the corresponding curves were constructed (Fig. 2d) with a mobile phase composed of more than 75% organic modifier. Such conditions can be called *liquid-like*, as there is still a portion of CO_2. Nonetheless, a very similar behavior to UHPLC is expected, similarly to what was previously observed with maleic acid. In this case, the beneficial use of high temperatures is even easier to understand, compared to low temperatures where an extreme loss of kinetic performance was seen already at 15 °C (Table 1). High temperatures were again very helpful in keeping the C-term dominating region of the VD plot flatter, while at 15 °C and less, an immediate loss of kinetic performance was observed right after the u_{opt}. At −5 °C, the situation becomes even more extreme, with a severe loss of plate count and large minimum plate height values (Table 1). Based on these observations, it becomes fairly easy to understand that UHPSFC analyses at temperature close to 0 °C have very limited interest, at least from a kinetic performance point of view, when the separation requires high amount of co-solvent in the mobile phase. On the contrary, high temperature should be preferentially used to achieve high kinetic performance, when using a highly organic mobile phase.

3.2. Pressure plots evaluation

Besides the plate height curves, pressure plots were also constructed. As previously discussed, the VD plots at elevated temperature generally showed a favorable *C*-term in most of the tested analytical conditions. Therefore, the use of high linear velocities is possible without experiencing an important peak broadening. However, even the latest generation of UHPSFC instruments does not have the same pressure range as UHPLC systems. Considering an ABPR value of 12–15 MPa, the UHPSFC system employed in the present work can tolerate only up to 25–30 MPa from the column itself. Consequently, such constraint must be taken into consideration when altering the column temperature during method development. To properly define the situation, pressure plots were collected, using the same analytical conditions as for the plate height curves (Fig. 3a–e). The contribution originating from the ABPR module (12 MPa) was systematically subtracted, to have only

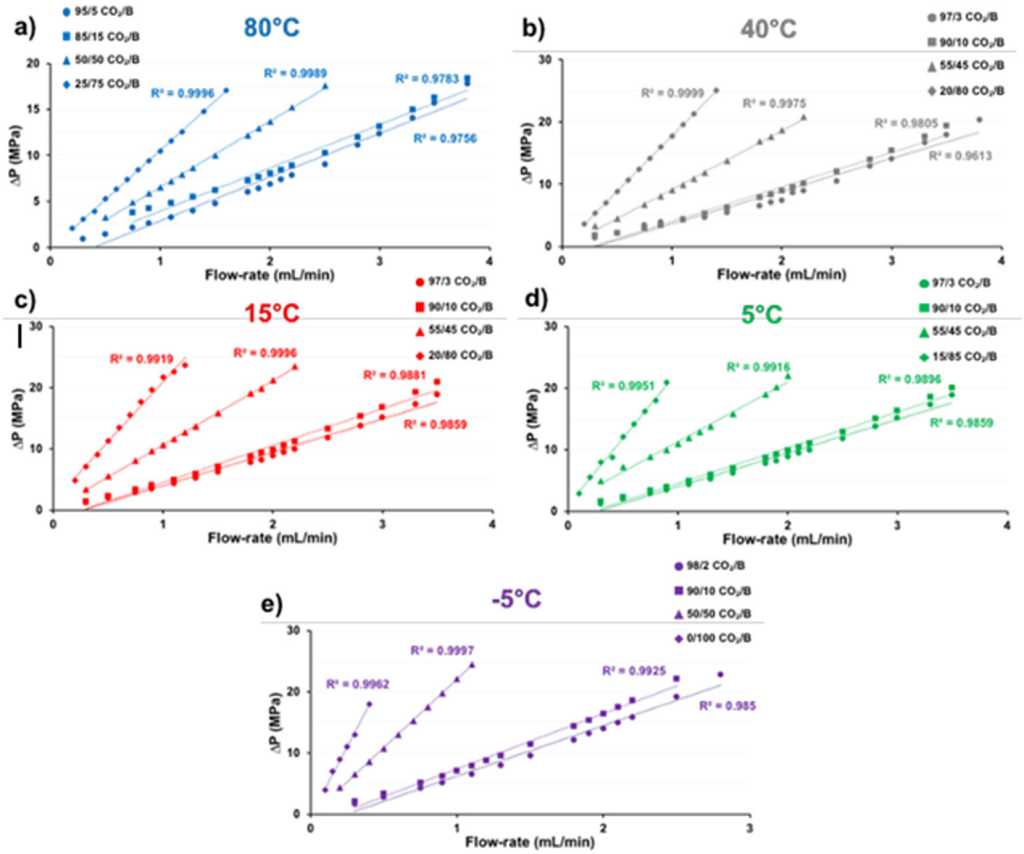

Fig. 3. Pressure plots recorded under isocratic conditions at different conditions: a) 5% - 80 °C (circles), 15% - 80 °C (squares), 50% - 80 °C (triangles), 75% - 80 °C (rhombi); b) 3% - 40 °C (circles), 10% - 40 °C (squares), 45% - 40 °C (triangles), 80% - 40 °C (rhombi); c) 3% - 15 °C (circles), 10% - 15 °C (squares), 45% - 15 °C (triangles), 80% - 15 °C (rhombi); d) 3% - 5 °C (circles), 10% - 5 °C (squares), 45% - 5 °C (triangles), 85% - 5 °C (rhombi); e) 2% to −5 °C (circles), 10% to −5 °C (squares), 50% to −5 °C (triangles), 100% to −5 °C (rhombi).

column pressure values. The data points out quite evidently that the increase in column backpressure at low temperature is important, but it allows to normally perform analyses at a flow-rate of 1.50–1.80 mL min^{-1} with 100 × 3 mm I.D. columns without suffering from overpressure, even with ABPR values up to 150 bar and a co-solvent percentage reaching 10–15% in the mobile phase (Fig. 3d and e).

Nonetheless, the situation becomes more challenging as soon as higher percentages of organic modifier are employed. At 50% of co-solvent, column temperatures down to 5 °C still allow analyses in the flow-rate range of 1.50–1.80 mL min^{-1} (Fig. 3d). At −5 °C, however, the limit has to be set at 1.00 mL min^{-1}. This impediment should not pose any issues in terms of peak broadening, as the H_{min} found in the VD plots is reached at relatively low linear velocities under these extreme conditions. The main problem revolves around the obvious increase in analysis time, and this drawback should be taken into consideration during the method development process. While at relatively high temperatures (40 °C – Fig. 3b) the employment of high amounts of co-solvent, equal or above 70%, was still possible with relatively high flow rates (1.20–1.50 mL min^{-1}), at −5 °C the situation becomes quite extreme. With these conditions, flow rates values above 0.30–0.40 mL min^{-1} already generate over 20 MPa (total backpressure of 32 MPa with an ABPR of 12 MPa), as seen in Fig. 3e. The combination of high column backpressure and the poor kinetic performance witnessed in Fig. 2d does not suggest once more the use of such low temperature when high co-solvent percentages (above 75%) are required during method development.

3.3. Thermodynamic evaluation: van't hoff plots

Column temperature has not only an important impact on the kinetic aspect of a chromatographic method, but also on its thermodynamic behavior (retention profiles and selectivity). In LC, an increase in temperature is associated with a reduction of retention for a given compound. Once again, the situation differs in UHPSFC, due to the peculiar nature of the mobile phase. With a supercritical mobile phase, a temperature increase generally provides an increase in retention. This behavior, opposite to the one observed in LC, is the result of the important changes in the mobile phase density since higher temperature reduces the mobile phase density. This phenomenon is well known for a super/subcritical mobile phase with a limited percentage of organic modifier. However, the behavior may be quite different if larger amounts, especially above 50%, are used in the mobile phase. Hence, the retention profiles of the four representative compounds previously used have been studied via the use of van't Hoff plots. In this case, six column temperatures from −5 to 80 °C were considered and a constant flow-rate was applied (Fig. 4a). For butylparaben, which is eluted under supercritical conditions, the impact of column temperature follows the expected behavior, as low temperatures (right side of the van't Hoff plot) cause a significant decrease of retention. On the other hand, indoxyl sulphate follows a typical LC trend, with a decrease of retention at higher temperatures. An interesting pattern was noticed for both uracil and maleic acid. These two analytes were eluted in subcritical conditions, with different percentages of organic modifier (15% for uracil and 45% for maleic acid). These compounds exhibited a peculiar behavior: from 40 °C to 80 °C, their retention increased, which resembles the same as for butylparaben. However, the transition to low temperatures (from 40 °C to −5 °C), was accompanied by an increase in the retention typical of an LC-like behavior. The van't Hoff plots, therefore, possess a characteristic U-shape, in which both the use of high and low temperatures translates into higher retention factors. These U-shaped plots, which have been previously witnessed with a chiral stationary phases [34], are confirmed also for achiral separations with a stationary phase employing sub-2 μm silica particles.

To avoid any influence on the analytes retention by the column backpressure, the van't Hoff plots have also been measured, in a second occasion, also under isobaric conditions. To do so, a fixed column backpressure value of approximately 30 MPa was applied for all conditions. These van't Hoff plots (Fig. 4b) confirmed the same trends as the ones previously observed under pure isocratic conditions.

To conclude, under subcritical conditions, analytes show a different retention model to those observed in either supercritical or liquid-like conditions. More importantly, it highlights the potential of testing different column temperatures during the method development process to adjust selectivity. As clearly shown in Fig. 4a and b, the four analytes describe three different retention profiles. At either very high or very low temperatures, these differences are maximized. These changes in the retention profiles might be used, therefore, to obtain an improved separation, without the need to adjust neither the mobile phase gradient nor its composition nor changing the stationary phase.

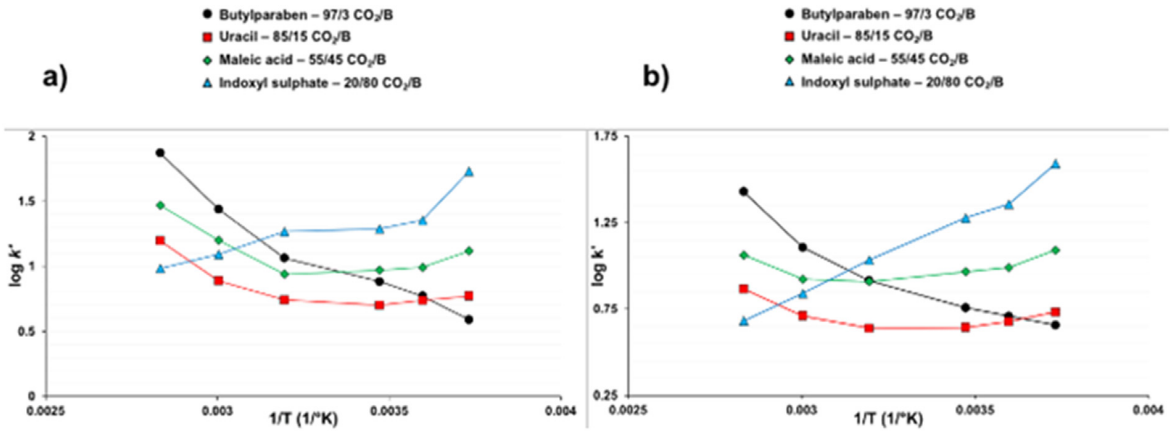

Fig. 4. Van't Hoff plots obtained, under a) isocratic and b) isopycnic conditions over six temperatures (80 °C–60 °C – 40 °C–15 °C – 5 °C and −5 °C), for a set of 4 compounds (butylparaben, uracil, maleic acid, indoxyl sulphate) eluted each at different percentages of cosolvent: 3% for butylparaben, 15% for uracil, 45% for maleic acid and 80% for indoxyl sulphate. Cosolvent used: MeOH/H_2O 95/5 *v/v* (+10 mM of ammonium formate for maleic acid and indoxyl sulphate).

3.4. Evaluation of the stationary phase stability at elevated temperature

Next to the theoretical aspects of tuning temperature in SFC, some practical aspects were also investigated, such as the stationary phase stability at high temperatures (>40 °C). A quick analysis of the applications developed for UHPSFC indicate that column temperatures rarely exceeded 50–70 °C [23,35–37], although column manufacturers indicate a good robustness at temperatures even above 60 °C under UHPSFC conditions. This claim derives from the absence (or the presence of very limited amounts) of water in the mobile phase. Therefore, it was decided to verify the stability of the column employed in this study (Torus 2-PIC) up to a temperature of 80 °C. The column was left for 3 days at 80 °C with the mobile phase continuously flowing through the stationary phase. At the beginning, three subsequent injections of a mixture of probe compounds (i.e. butylparaben, uracil, caffeine and adenosine) were performed under generic gradient mode. After these three initial analyses, the mobile phase was set to generic isocratic conditions (80:20 CO_2:modifier *v/v*) at a flow-rate of 2 mL min^{-1} for 1 h. Then, three injections of the probe compounds mixture were performed again, followed once more by the waiting period with the isocratic conditions. Such cycle was repeated for three days, to reach above 10′000 column volumes percolated through the column. In Fig. 5, the chromatograms obtained at different time points were reported. The chromatograms do not show important loss in performance even after exposure at 80 °C for more than 10′000 column volumes. In Fig. S3, the evolution of the retention times, peak widths and pressure drop were presented. The retention times for the four analytes do not change significantly after a prolonged exposure at 80 °C (Fig. S3a). This suggests that the column was able to keep the same retention profile under such harsh conditions. Moreover, the relative standard deviations (RSD%) calculated on the apparent retention factors in gradient mode for the four model compounds (*k**) present a very low variability over this extended period of time, with values ranging between 2 and 2.2%. A similar evaluation was made for peak widths recorded at half-heights ($w_{50\%}$), and only minimal variations (below ± 5%) were observed (Fig. S3b). Finally, the inlet pressure values registered throughout the study were also constant (Fig. S3c). This excellent chromatographic behavior at elevated temperature can be attributed to the use of a specifically designed column made with BEH (bridge ethyl hybrid) particles rather than classical silica. BEH particles have indeed already been shown to be highly resistant to elevated temperature in LC as well [38]. In addition, the absence of water in the mobile phase under the selected conditions was also highly beneficial for column stability, even under extreme temperature conditions, as it avoids the silica dissolution phenomenon [39,40]. It can be concluded, therefore, that the use of elevated temperatures in UHPSFC is not detrimental for the stationary phase stability. Obviously, such conclusion cannot be drawn for other UHPSFC columns, and there will be a need to evaluate other stationary phases and silica particles.

3.5. Applications of SFC at high or low column temperatures

3.5.1. Advantages of high temperatures for practical applications

In this section, some potential applications highlighting the advantages to work at elevated temperature are described. Fig. 6 shows the separation of two corticosteroid medications, namely dexamethasone and betamethasone, under UHPSFC conditions at four different column temperatures (80 °C, 40 °C, 5 °C and −5 °C). These two compounds are diastereomers, as they only differ in the position of the methyl group attached on the 16th carbon atom of the steroidal core structure (Table S1). The high degree of similarity between these two drugs makes it hard to obtain a proper separation using generic conditions at a temperature of 40 °C on the selected stationary phase. The example shows that the use of low temperatures was not beneficial for the separation. The transition from 40 °C to 5 °C resulted in a reduction of resolution, while the passage to *sub-zero* conditions caused a complete loss of resolution of both corticosteroids. On the other hand, an improvement was observed at higher temperature (80 °C). These two corticosteroids were eluted with a relatively important percentage of co-solvent (around 30%). It is finally important to notice that the retention increases at −5 °C in Fig. 6, while it should normally decrease at

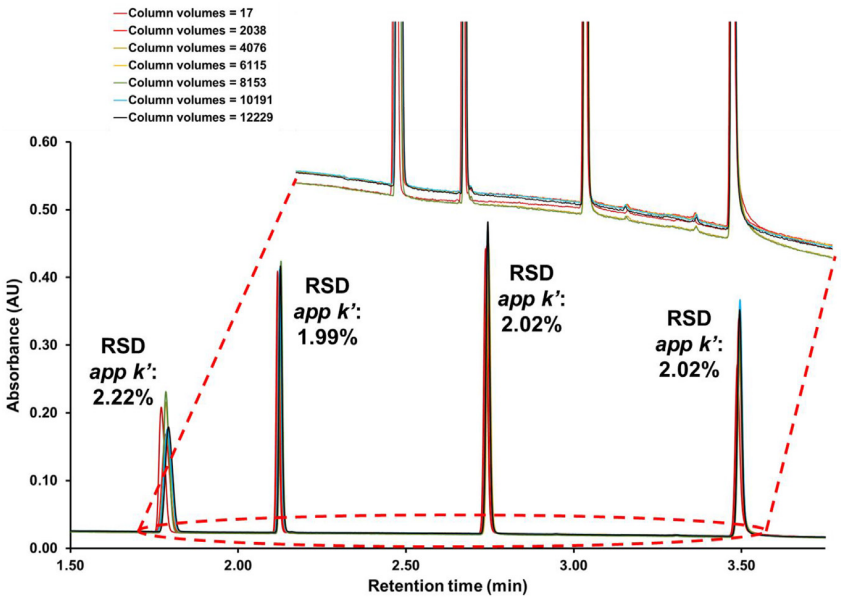

Fig. 5. Overlay of seven chromatograms of a mixture of caffeine, butylparaben, uracil and adenosine at different column volumes flushed during the three days of analysis at 80 °C. Column: Torus 2-PIC 100 × 3.0 mm I.D. 1.7 μm. Detection: UV at 254 nm.

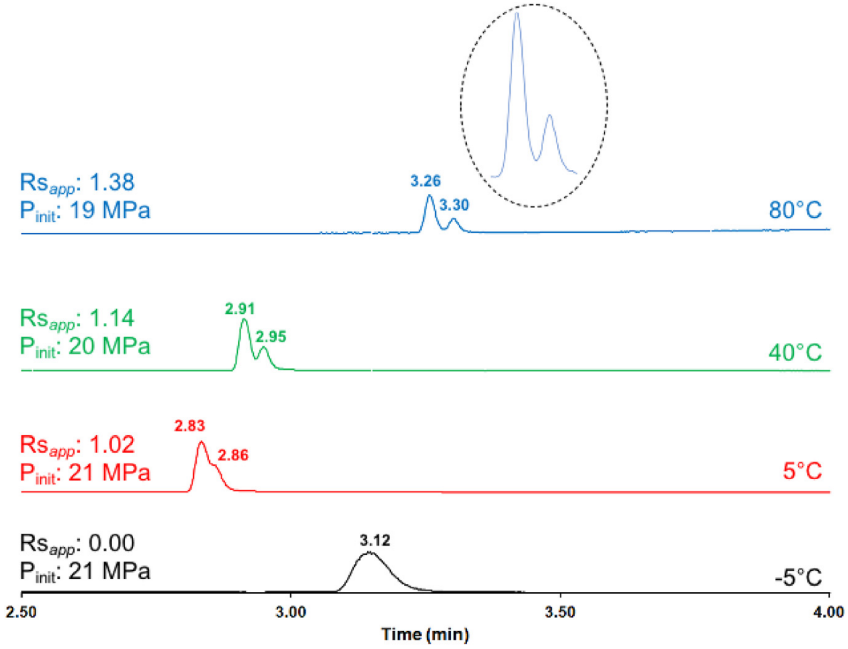

Fig. 6. Overlay of chromatograms obtained for the analytes dexamethasone + betamethasone at four different temperatures (80 °C–40 °C – 5 °C and −5 °C). Column: Torus 2-PIC 100 × 3.0 mm I.D. 1.7 μm. Detection: SIR at 393 *m/z*.

lower temperature. This change is due mostly to the different flow-rate used in the external oven configuration (1.20 mL min^{-1}) compared to the one with the standard configuration (1.50 mL min^{-1}). Moreover, the external oven setup uses a cooling liquid to ensure the *sub-zero* environment, while the normal oven uses air to transmit heat, which might also cause differences in the chromatographic behavior observed.

A second example highlighting the benefits of elevated temperatures is shown in Fig. 7, where a mixture of seven closely-related steroids with identical molecular weights was injected. The seven steroids all belong to the family of hydroxytestosterone, being either positional isomers or diastereomers. In Fig. 7, the overlapped chromatograms recorded at 80 °C, 40 °C, 5 °C and −5 °C are presented. Similarly, to the previous example, the use of high temperatures was relevant to obtain the highest resolution, up to six peaks have been partially resolved at 80 °C. Only 2α and 2β-hydroxytestosterone, were very slightly separated at −5 °C (according to MS identification), while they completely co-eluted at higher temperatures. These examples point a general benefit of employing high temperatures to obtain better chromatographic results. Nonetheless, more applications should be developed to further support such hypothesis.

3.5.2. *Advantages of low temperatures for practical applications*

In the previous section, the benefits of employing high temperatures have been illustrated. With compounds eluted at relatively high percentage of co-solvent (30% or above), increasing the column temperature has proven to be quite useful in tuning the resolution between peaks. However, such strategy is not always the best option to increase the selectivity. With some applications, the

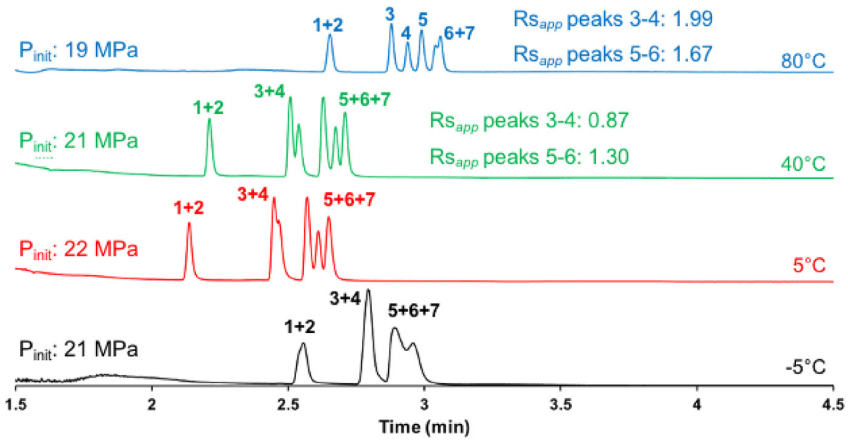

Fig. 7. Overlay of chromatograms for a mixture of seven steroids (1 + 2: 2α/β-hydroxytestosterone; 3: 19-hydroxytestosterone; 4: 6α-hydroxytestosterone; 5: 15β-hydroxytestosterone; 6: 11β-hydroxytestosterone; 7: 2α-hydroxytestosterone; 8: 16α-hydroxytestosterone) at four different temperatures (80 °C–40 °C – 5 °C and −5 °C). Column: Torus 2-PIC 100 × 3.0 mm I.D. 1.7 μm. Detection: SIR at 305 *m/z*.

lowering of temperatures can be beneficial, instead. In Fig. 8, the chromatograms obtained for (E/Z)-endoxifen at different temperatures (80 °C to −5 °C) are reported. This example is quite demonstrative of the potential advantages in the use of very low column temperatures, since the separation of the two isomers, namely E-endoxifen and Z-endoxifen, was extremely poor at 40 °C (Rs < 1). The temperature increase, up to 80 °C, did not improve the situation, as both analytes presented a total co-elution. Instead, at 5 °C and, more importantly, at −5 °C, the separation appeared to be largely improved (Rs of 1.1 at −5 °C). In this case, the two isomers were eluted in a range of co-solvent around 45–50%. Despite the lower kinetic performance, a better resolution was obtained at the lowest temperature. The analytes present a double bond between two carbon atoms; which is known to be quite rigid due to the presence of π electrons. The temperature lowering, diminishes the available energy in the system. The double bond becomes more rigid, reducing the rotation phenomenon and thus enabling a better stability of the two isomers. At high temperature, such small rotations become more feasible, making the separation of the E/Z isomers harder to accomplish. Consequently, it becomes easier for the stationary phase to perform at least a partial separation of the molecules, which was almost impossible at 40 °C or higher temperatures.

3.6. Impact of temperature on UV sensitivity

In UHPSFC, it is known that temperature may affect the sensitivity of UV detection. Therefore, it was interesting to study whether varying temperature in a wide range could indeed significantly affect UV sensitivity. In UHPSFC, UV detection is always more problematic and less sensitive than in LC [41,42]. There are mostly two reasons that could explain the lower sensitivity. First, pressure changes can induce density fluctuation, which eventually disturbs the UV signal and increases the background noise [41,42]. Secondly, refractive index may change as the mixture between supercritical CO_2 and the organic modifier varies along the gradient profile [41,42]. The refractive index is known to be impacted also by changes in temperature and pressure [43]. The development of UHPSFC instruments has greatly addressed this issue and UV sensitivity has been significantly improved compared to the previous generation of SFC instruments. Nonetheless, any changes in the parameters that control the state of the mobile phase may negatively impact the UV performance. Moreover, it is also important to consider that the eluent will experience important changes in the temperature profile during a separation. These shifts in the mobile phase temperature may result in increased background noise with a consequent decrease of the method sensitivity. To verify if the use of high or low temperatures might induce some important changes in sensitivity in SFC, a mixture containing a commercially available API, paroxetine hydrochloride, with three of its known pharmacopoeia impurities (A, C and H) were injected at four column temperatures (80 °C, 40 °C, 5 °C and −5 °C) (Fig. 9). The mixture was prepared to have, a signal-to-noise (S/N) ratio of approximately 10 for the three impurities at 40 °C. As reported in Fig. 9, the calculated S/N values at 80 °C, 5 °C and −5 °C were very close to those obtained at 40 °C, and the required S/N ratio of at least 10 was met under all conditions for the same mixture. This might suggest that the mobile phase mixture was effectively kept homogeneous under all temperature conditions.

4. Conclusions

The influence of column temperature range, varying from 80 °C down to −5 °C, on chromatographic performance was studied in UHPSFC. First, a kinetic performance evaluation was made for a wide range of mobile phase compositions, from an almost supercritical state (3% of cosolvent) to a liquid-like condition (80% of modifier). The results have pointed out that, with a UHPSFC mobile phase with weak proportions of organic modifier, the use of low column temperatures, even below 0 °C, does not translate into an important loss of kinetic performance. On the other hand, the use of elevated temperatures (up to 80 °C), seems to provide some interesting results, although it was not possible to clearly reach the van Deemter minima due to instrument limitation. . The increase in the co-solvent percentage, on the other hand, has inverted the tendencies. In this context, high temperatures (above 40 °C), are proved to be the best choice, from a kinetic performance point of view, for a percentage of co-solvent in the mobile phase equal to at least 45–50% and above. Low mobile phase temperatures, however, have shown a decrease of the H_{min} values. It is also important to mention that the upper pressure limit of the system (about 40 MPa) is a serious constraint while working with low temperatures and

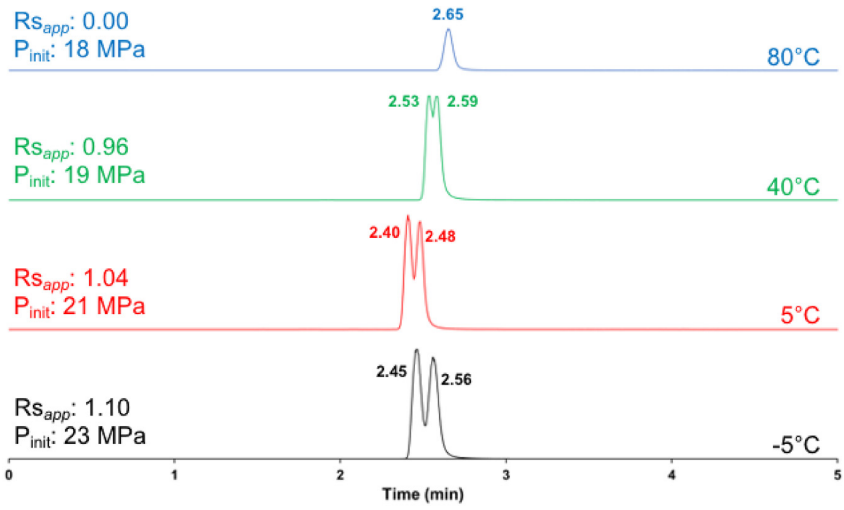

Fig. 8. Overlay of chromatograms obtained of the isomers E/Z-endoxifen at four different temperatures (80 °C–40 °C – 5 °C and −5 °C). Column: Torus 2-PIC 100 × 3.0 mm I.D. 1.7 μm. Detection: SIR at 374 *m/z*.

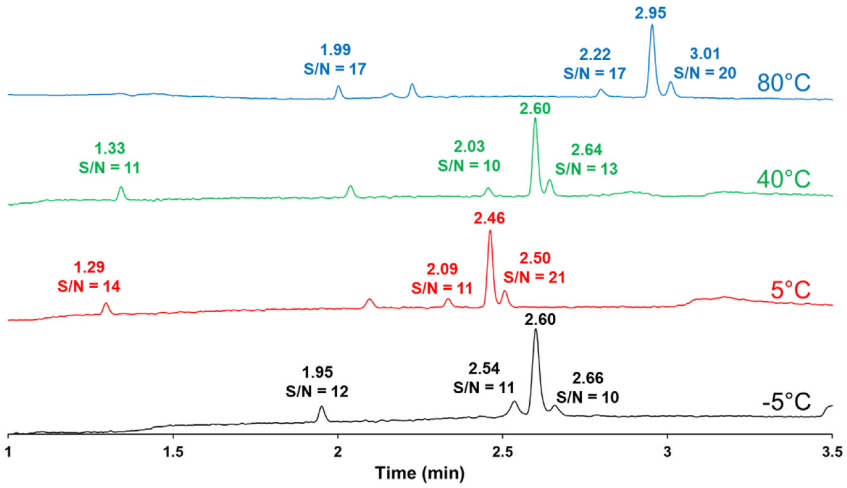

Fig. 9. Chromatograms obtained at four temperatures (80 °C–40 °C – 5 °C and −5 °C) relative to the separation of paroxetine and its impurities A, C and H, diluted at a concentration needed to register a S/N value of approximately 10. Column: Torus 2-PIC 100 × 3.0 mm 1.7 μm. Detection: UV at 214 nm.

high proportion of organic modifier. In terms of retention profiles (van't Hoff plots), the experimental results have produced interesting trends, according to the mobile phase composition. Under supercritical and liquid-like conditions, compounds have shown opposite retention profiles, while under a subcritical state, the Van't Hoff plots demonstrated a U-shaped profile, meaning that similar retention factors can be obtained either at high or low temperatures.

The stability of the Torus 2-PIC stationary phase was also evaluated at 80 °C, and the column demonstrated an excellent stability over time (>10000 column volumes) in terms of retention times and peak shapes. Different classes of closely related compounds, challenging to resolve at standard temperature, were better separated either at higher or lower column temperatures. This result seems to indicate that the tuning of mobile phase temperature can be a useful variable to optimize a chromatographic separation, nonetheless further applications are needed to strengthen this claim. Finally, an assessment of the UV performance has shown that low or high temperature do not generate any loss in terms of sensitivity compared to the common column temperature.

These results provide an update on the current knowledge on the impact of column temperature on modern SFC, taking also into account the latest developments in terms of mobile phase composition and gradient profiles. The use of unconventional column temperatures has proven to be valuable to tune the resolution profile of challenging mixtures of analytes. Such parameter should not be immediately discarded but deserves better consideration in the method development process.

CRediT authorship contribution statement

Gioacchino Luca Losacco: Writing - original draft, Methodology, Investigation. **Szabolcs Fekete:** Supervision, Writing - review & editing. **Jean-Luc Veuthey:** Supervision, Resources, Writing - review & editing. **Davy Guillarme:** Supervision, Writing - review & editing, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2020.07.076.

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II.4 Applicability of UHPSFC-MS to metabolomics

II.3.1 Optimization of separation condition for the simultaneous analysis of apolar and polar metabolites

As previously discussed in section II.3, the introduction of UC-type gradients has enabled UHPSFC to potentially extend its applicability range, as highly polar compounds may be eluted and, thus, detected. UHPSFC, therefore, might be considered not only an alternative to NPLC and RPLC, but also to HILIC. In this context, simultaneous analyses of both lipophilic and hydrophilic substances can be theoretically feasible using UHPSFC, while it is not the case with LC techniques.

In this article, the implementation of UHPSFC, coupled to a tandem MS, was evaluated for the analysis of 57 endogenous metabolites in neat solutions. The focus of this work has been put in optimizing the chromatographic conditions, to have the largest number of metabolites detected.

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Applicability of supercritical fluid chromatography – mass spectrometry to metabolomics. I – Optimization of separation conditions for the simultaneous analysis of hydrophilic and lipophilic substances

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ABSTRACT

The aim of this study was to evaluate the suitability of SFC-MS for the analysis of a wide range of compounds including lipophilic and highly hydrophilic substances (log P values comprised between −6 and 11), for its potential application toward human metabolomics. For this purpose, a generic unified chromatography gradient from 2 to 100% organic modifier in CO_2 was systematically applied. In terms of chemistry, the best stationary phases for this application were found to be the Agilent Poroshell HILIC (bare silica) and Macherey-Nagel Nucleoshell HILIC (silica bonded with a zwitterionic ligand). To avoid system overpressure at very high organic modifier proportion, columns of 100 × 3 mm I.D. packed with sub-3 μm superficially porous particles were selected. In terms of organic modifier, a mixture of 95% MeOH and 5% water was selected, with 50 mM ammonium formate and 1 mM ammonium fluoride, to afford good solubility of analytes in the mobile phase, limited retention for the most hydrophilic metabolites and suitable peak shapes of ionizable species. A sample diluent containing 50%ACN/50% water was employed as injection solvent.

These conditions were applied to a representative set of metabolites belonging to nucleosides, nucleotides, small organic acids, small bases, sulfated/sulfonated metabolites, poly-alcohols, lipid related substances, quaternary ammonium metabolites, phosphate-based substances, carbohydrates and amino acids. Among all these metabolites, 65% of the compounds were adequately analyzed with excellent peak shape, 23% provided distorted peak shapes, while only 12% were not detected (mostly metabolites having several phosphate or several carboxylic acid groups).

1. Introduction

Metabolomics is defined as the systematic identification and quantification of small molecules known as metabolites, produced by the metabolism of living organisms, in different biological fluids [1]. It generally requires the use of highly powerful analytical techniques, such as nuclear magnetic resonance (NMR) spectroscopy or mass spectrometry (MS), the latter usually coupled to different chromatographic techniques [1]. The interest on metabolomics has rapidly increased among scientists since it represents a valuable approach to perform clinical diagnosis in precision medicine [2,3]. There are, however, several issues linked to metabolomic analysis. In particular, it implies the analysis of an extremely wide range of molecules, possessing quite diverse physico-chemical properties [1,3]. Currently, the state-of-the-art approach consists in combining reversed phase liquid chromatography (RPLC) and hydrophilic interaction chromatography (HILIC) with high-resolution MS instruments, such as quadrupole-time-of-flight or quadrupole-Orbitrap MS [4]. These two chromatographic approaches present some challenges. Indeed, RPLC is not ideal to analyze the most polar metabolites, due to the low retention of such

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analytes on classical RPLC stationary phases (C_4, C_8 or C_{18}) [5,6]. In HILIC mode, polar metabolites can be sufficiently retained [7,8], but the technique is not adapted to the most lipophilic substances, and remains sometimes difficult to use (i.e. lack of repeatability, significant impact of sample diluent, complex and multiple retention mechanisms, etc.) [9,10].

In this context, supercritical fluid chromatography (SFC), coupled to MS, has potential as an interesting alternative to RPLC and HILIC. Initially, SFC was developed as a substitute to normal phase liquid chromatography (NPLC) [11], for the analysis of highly hydrophobic substances, such as lipids or petrochemical samples, due to the non-polar characteristics of CO_2. SFC was, also, used instead of liquid chromatography for compounds of pharmaceutical interest in both academia [12] and industry [13,14], as well as in other processes such as impurity control [15] More recently, SFC has been employed for the analysis of substances with increasing polarity [16–21], thanks to the addition of polar organic modifier and salts in the mobile phase. For instance, Taguchi et al. applied SFC for the simultaneous analysis of liposoluble and hydrosoluble vitamins [22]. In their work, the authors demonstrated that compounds with log *P* values between −2.1 and 10.1 can be analyzed in the same run with a generic SFC gradient varying from pure CO_2 to pure MeOH. The suggested designation for such a gradient was unified chromatography (UC), as it was able to make the link between supercritical conditions at the beginning of the gradient and liquid chromatography at the end. Moreover, SFC is known to be fully compatible with MS, often resulting in improved sensitivity compared to RPLC, thanks to the presence of solvents with a low surface tension in the mobile phase (i.e. methanol) and the absence (or limited presence) of water [23–25]. It also possesses a different behavior towards matrix effects than LC–MS, showing predominantly ion suppression while, in LC–MS, ion enhancement is more common [25–27].

The aim of this study was to evaluate the applicability of SFC-MS to the field of metabolomics and to find out some generic SFC conditions applicable to a training set of 57 representative metabolites, covering a broad range of log *P*, from −6 to 11. For this purpose, a comparison of various column chemistries as well as mobile phase conditions (organic solvent choice and composition, additives nature and concentration, temperature, etc.) was carried out. An evaluation of the kinetic performance and the behavior of the SFC-MS interface at high percentage of co-solvent was also studied.

2. Material and methods

2.1. Chemicals, reagents and columns

All metabolites, reported in Table S1 of the supplementary material, were obtained from Sigma-Aldrich (Buchs, Switzerland). These metabolites were selected based on the human metabolome database (HMDB) [28], which contains about 100,000 entries representing a varied selection of human metabolites belonging to various chemical classes. In the present work, 57 representative metabolites were selected, based on their availability, price, diversity and representability of all the HMDB chemical classes. The chemical classes covered by the selected metabolites include sulfated/sulfonated metabolites, nucleosides, nucleotides, small organic acids, small bases, poly-alcohols, lipid related substances, quaternary ammonium metabolites, phosphate-based substances, carbohydrates and amino acids.

Methanol (MeOH), acetonitrile (ACN), isopropanol of OPTIMA LC/MS grade and water of UHPLC grade were purchased from Fisher Scientific (Loughborough, UK). Ammonium formate (AmF), ammonium acetate and ammonium fluoride (NH_4F) were purchased from Sigma-Aldrich. Pressurized carbon dioxide (CO_2) 3.0 grade (99.9%) was purchased from PanGas (Dagmerstellen, Switzerland). Seven different columns were compared, namely Poroshell HILIC 2.7 μm (Agilent, Santa Clara, CA, USA), Nucleoshell HILIC 2.7 μm (Macherey-Nagel, Düren, Germany), Sunshell Diol 2.6 μm and Sunshell 2-EP 2.6 μm (ChromaNik Technologies INC., Osaka, Japan), Cosmosil 3-hydrophenyl 2.5 μm (Nacalai Tesque INC., Kyoto, Japan), Kinetex C18 Polar 2.6 μm and Synergi Polar 2.5 μm (Phenomenex, Torrance, CA, USA). For sake of comparison, all the selected columns were of the same dimensions (100 × 3.0 mm).

2.2. UHPSFC-MS/MS instrumentation

All experiments were performed on a Waters Acquity UPC^2 system (Waters, Milford, MA, USA) equipped with a Binary Solvent Manager delivery pump, a Sample Manager autosampler which included a 10 μL loop for partial loop injection, a column oven and a two-step (active and passive) backpressure regulator (BPR). Acetonitrile and a mixture of MeOH/H_2O 50/50 were used as the weak and strong wash solvents, respectively, with volumes of 600 μL and 200 μL. The chromatographic system was hyphenated to a Waters TQD triple quadrupole via a double-T splitter interface from Waters. The hyphenation interface and splitter for UHPSFC-MS/MS were described elsewhere [29]. Additional make-up solvent for SFC-MS operation was brought to the system by a Waters Isocratic Solvent Manager (ISM) pump, delivering pure MeOH at 0.3 mL/min.

The TQD detector was operated in both positive and negative electrospray ionization (ESI) modes and the different parameters were optimized to obtain the highest sensitivity: source temperature at 150 °C, desolvation temperature at 450 °C, capillary voltage at 3.0 kV. Nitrogen was used as a desolvation gas at 600 L/hr, while argon was used as a collision gas at 0.10 mL/min. Other parameters such as cone voltages and collision energies were tuned, depending on the analyte, in a range of 10–40 V and 5–20 eV, respectively, as described in Table S1. Dwell times were set up between 8 and 20 ms, depending on the samples, to have a sufficient number of data points across each chromatographic peak. MassLynx 4.1 software was used for instrument control and data acquisition.

2.3. Sample preparation

Sample stock solutions were prepared by dissolving the selected compounds in a suitable sample diluent, according to their physicochemical properties. As reported in Table S1, either water, 0.1N NaOH in water, 0.1N HCl in water, MeOH, ethanol (EtOH), isopropanol (IPA), tetrahydrofuran (THF), or chloroform ($CHCl_3$) were used, to have all the stock solutions at a final concentration of 1 mg/mL. Stock solutions were then stored at −22 °C. Prior to injection, stock solutions were thawed and samples were diluted to a concentration of 20 μg/mL, by adding 980 μL of the solvent used in the stock solutions to 20 μL of sample.

Six mixtures were prepared (Table S2), with each molecule at a suitable concentration, to have comparable signal to noise ratios. The mixtures were diluted to a final volume of 1 mL.

2.4. Chromatographic conditions

The initial composition of the mobile phase was 98% CO_2/2% organic modifier, and was held constant for 1 min, with a subsequent 5-min linear gradient up to a final composition of 100% organic modifier. After an isocratic step of 1 min at 100% organic modifier, the column was reconditioned with initial conditions for 1.5 min. Back-pressure was maintained constant at 120 bar, while mobile phase temperature was kept at 40 °C. All compounds were

injected on all columns with an injection volume of 1 μL, either individually or as mixtures.

3. Results and discussion

3.1. Kinetic evaluation of SFC with unified chromatography gradient

Recently, the popularity of SFC has strongly increased thanks to the commercialization of modern instrumentation, compatible with columns packed with sub-2 μm particles, and a significant number of recent SFC applications have been reported with such columns. However, the generated backpressure remains high [15,30–32], even with supercritical or subcritical fluids, while the upper pressure limits of UHPSFC systems are more limited than those of UHPLC. For this reason, the amount of co-solvent in the mobile phase has to be kept in a reasonable range, to maintain optimal kinetic performance. A typical UHPSFC gradient ranges from 2 to 40% co-solvent, which is adequate to elute highly lipophilic and moderately polar compounds. However, SFC has also proven its applicability to the analysis of more polar compounds, and a new trend appeared, with an increase of the percentage of co-solvent up to 100% during the gradient. Under such conditions, the use of columns packed with sub-2 μm particles cannot be envisioned. Indeed, the elevated backpressure generated by high co-solvent percentage in the mobile phase would urge SFC users to apply relatively low flow rates. As an example, the experimental backpressures observed with different mobile phase conditions when using a 100 × 3.0 mm column packed with 1.7 μm particles were reported in Fig. 1A. On our UHPSFC system (Waters Acquity UPC^2), the upper system pressure limit (approximately 400 bar) was reached at 2.8 and 2.0 mL/min with pure CO_2 and 25% MeOH in CO_2, respectively. These flow rates are fully acceptable for a generic UHPSFC method, taking into account column dimensions. However, when the percentage of MeOH was further increased, the flow rate should be greatly reduced to maintain a reasonable backpressure. As an example, when using a mobile phase composed of 75% MeOH in CO_2, the flow rate was limited to 0.8 mL/min. One attractive solution to reduce the pressure involves the use of columns packed with superficially porous particles (SPP), also known as core-shell or fused-core technology. Thanks to their particular shape, these particles, with a diameter of c.a. 2.6 μm, are able to reach comparable efficiencies to fully porous sub-2 μm particles, but at lower backpressure. The experimental backpressures observed with the same mobile phase compositions as in Fig. 1A, when using a 100 × 3.0 mm column packed with 2.6 μm SPP, were reported in Fig. 1B. The difference was obvious, and a flow rate of 1.4 mL/min could now be reached, even with pure MeOH as mobile phase. In conclusion, the use of such columns represents a viable and interesting solution when unified gradients from 2 to 100% co-solvent have to be applied in SFC.

SFC has always been recognized for its excellent kinetic performance [33]. Indeed, due to the reduction of mobile phase viscosity and improvement of diffusion coefficients compared to liquid conditions, both optimal linear velocity and mass transfer are drastically enhanced compared to RPLC [34]. This is true in supercritical or subcritical conditions, but the kinetic performance with higher proportion of co-solvent has to be evaluated, since it has not been yet reported in the literature. For this purpose, four different metabolites were analyzed under isocratic conditions at different co-solvent percentages on the same column, namely Nucleoshell HILIC. On this column, a retention factor of 5 was obtained when analyzing caffeine, melatonine, maleic acid and indoxyl sulphate at 2, 18, 40 and 80% of co-solvent in CO_2, respectively. The corresponding Van Deemter curves were then constructed for each compound (see Fig. 2). In pure SFC conditions (case of caffeine), the shape of the curve was expected, with a relatively high optimal linear velocity of 7–8 mm/s, and a flat curve due to a limited contribution of the mass transfer resistance. In subcritical conditions (case of melatonin and maleic acid), the Van Deemter curve shapes were quite different. Indeed, the mobile phase viscosity and mass transfer resistance were increased due to the higher proportion of organic modifier in the mobile phase, and consequently, the optimal linear velocity decreased to 2–3 mm/s. This was even more noticeable for indoxyl sulphate, which was eluted with 80% co-solvent in CO_2 where the conditions could be considered as comparable to RPLC and the resistance to mass transfer was therefore greatly increased. The optimal linear velocity was also drastically reduced with an optimal value around 1 mm/s (corresponding to a flow rate of only 0.3 mL/min on a 3.0 mm internal diameter column). If we consider the simultaneous analysis of these four metabolites, a gradient ranging from a very low percentage (2%) to a high proportion of co-solvent (possibly 100%) has to be used. However, as previously discussed, the optimal linear velocity (or flow rate) is not the same for all these metabolites, which raises the question of the ideal flow rate in this particular situation. On the one hand, an elevated linear velocity is not adapted to the highly retained compounds eluted with high organic modifier proportion and would generate system overpressure. However, a low linear velocity is not favorable for weakly retained compounds (low kinetic performance, according to the van Deemter curves) and would increase analysis time. In consequence, the best compromise was to select an intermediate linear velocity between 3 and 4 mm/s, corresponding to a flow rate between 0.8 and 1 mL/min on a 3.0 mm I.D. column. Another interesting approach would have been to implement a decreasing flow rate gradient (simultaneously with the organic modifier gradient). In this way, a high flow rate could be used at the beginning of the gradient, to elute weakly retained metabolites. Then, the flow rate would be progressively decreased, in order to fit with the lower optimal linear velocity requirement observed at high co-solvent percentage, and to maintain a constant backpressure due to the increasing mobile phase viscosity to avoid system overpressure. This approach was not selected in order to keep generic conditions easily implemented and to avoid irreproducible retention times.

3.2. Behaviour of the SFC-MS interface at high co-solvent percentages

The nature and specificity of the available interfaces for hyphenating SFC and MS have been substantially studied [29,35,36]. Nowadays, the most prevalent and promising interface for electrospray ionization (ESI) operation seems to be the “pre-BPR splitter with sheath pump” interface [36] used in this study. However, the behavior of this interface was only described for generic SFC conditions with proportions of co-solvent lower than 50%. The main features of the interface that need to be evaluated are i) the amount of MeOH reaching the ionization source, ii) the split ratio between the flow going to the source and to the waste, and iii) the dilution factor induced by the additional make-up solvent in the interface. The calculations for a mobile phase containing an important fraction of co-solvent were made and results were graphically reported in Figs. S1 and S2 of the supplementary material. For more information about the calculations, the reader is referred to [36].

The flow-rate of the organic modifier reaching the ESI source with a given SFC-MS setup is an important parameter. If it is too high, the conditions may not be ideal for the spray formation in the ESI source. On the other hand, analytes precipitation can occur in the tubing from the interface to the MS source if this flow is too low, due to CO_2 decompression cooling (endothermic reaction). Generally, a flow rate of approximately 250 μL/min is considered to avoid both issues. At a reasonable co-solvent percentage of 20%

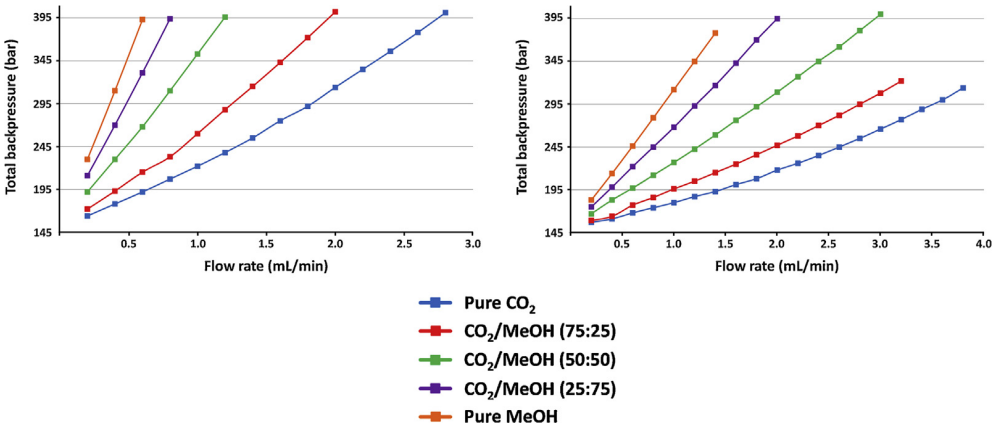


Fig. 1. Total backpressure as a function of the SFC pump flow rate for an Acquity UPC^2 Torus 2-PIC 1.7 μm, 100 × 3.0 mm column (A) and a Nucleoshell HILIC 2.7 μm, 100 × 3.0 mm column (B). Different mobile phase conditions are represented: pure CO_2 (blue), CO_2/MeOH 75:25 (red), CO_2/MeOH 50:50 (green), CO_2/MeOH 25:75 (purple) and pure MeOH (orange). Mobile phase temperature of 40 °C and backpressure of 150 bar. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

organic modifier, the flow entering the ESI source is highly dependent on the mobile phase and make-up solvent flow rates, and vary between 120 and 370 μL/min, as reported in Fig. S1A. Therefore, the make-up flow rate has to be carefully selected based on the SFC pump flow rate. This trend is reduced when the percentage of co-solvent is increased to 40% (see Fig. S1B). In these conditions, the flow rate entering the ESI source was comprised between 180 and 330 μL/min and the make-up flow rate was much less critical. Finally, with a higher co-solvent percentage (see Fig. S1C, corresponding to 80%), the flow-rate of the organic modifier entering the ESI source was completely leveled at around 270 μL/min (for MeOH). Under these conditions, the make-up solvent addition could be stopped to reduce solvent consumption. Nevertheless, the make-up solvent could still be maintained if its nature strongly differs from the one of the co-solvent, and if it could provide a sensitivity improvement [37]. Finally, in the case of unified chromatography gradient, the best solution would be to implement a decreasing solvent addition, to improve the ionization of the early-eluting metabolites and reduce solvent consumption during the elution of the strongly retained metabolites. In the present work, the latter approach was not selected, since it could have raised some issues regarding the normalization of the MS signals given by the compounds [38]. A make-up flow rate of 0.3 mL/min of MeOH was finally selected as the best compromise for unified chromatography gradient.

The dilution factor due to the SFC-MS geometry (relevant for concentration dependent detector, such as ESI/MS) was reported in Fig. S2 for a mobile phase containing 80% MeOH in CO_2. It is due to the addition of the make-up solvent, and only depends on the SFC and make-up pump flow rates. As mentioned in Section 3.1, when performing a unified chromatography gradient, the SFC pump flow rate should be reasonable (around 1 mL/min), to achieve the best compromise in terms of kinetic performance. Under such SFC flow rate conditions, the dilution factor remains below 2, whatever the make-up pump flow rate, which is fully acceptable. Moreover, if the flow rate of the additional pump was fixed at 0.3 mL/min (which was the case in the present study), the dilution factor was equal to only 1.3, and sensitivity loss due to dilution on the interface can then be considered as negligible.

Finally, the split ratio due to the "pre-BPR splitter with sheath pump" interface geometry, with unified chromatography gradient, was not discussed here, since it is only relevant for atmospheric pressure chemical ionization (APCI) source (mass dependent device), which was not used in this work.

3.3. Evaluation of stationary phase chemistries

There is a wide variety of columns available for SFC operation, and this parameter remains the most important for optimizing a separation. Even if the number of stationary phases specifically

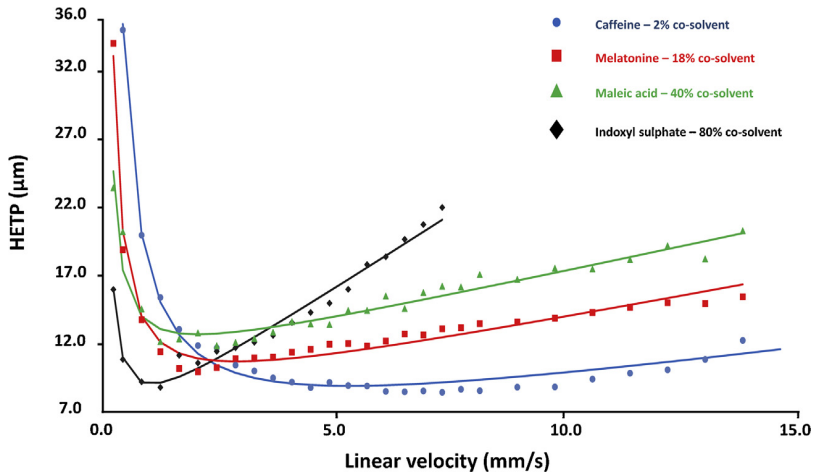


Fig. 2. Van Deemter curves for 4 different compounds: caffeine (2% co-solvent, blue dots), melatonine (18% co-solvent, red squares), maleic acid (40% co-solvent, green triangles), indoxyl sulphate (80% co-solvent, black diamonds). Mobile phase: CO_2 + 10 mM in MeOH/water (95:5). Mobile phase temperature of 40 °C and backpressure of 150 bar. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

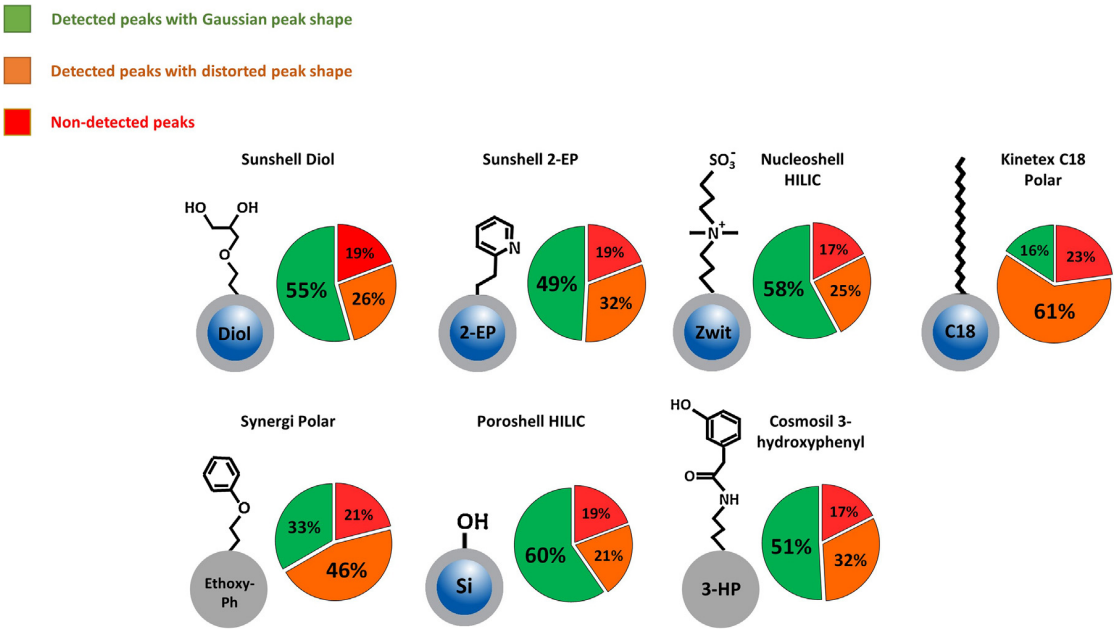


Fig. 3. Proportions of non-detected peaks (red), distorted peaks (orange) and Gaussian peaks (green) obtained on the seven tested stationary phases. Mobile phase: CO_2 + 10 mM in MeOH/water (95:5). Mobile phase temperature of 40 °C and backpressure of 120 bar. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

designed for SFC is restricted, columns dedicated to NPLC, RPLC and HILIC can also be successfully employed in SFC conditions [39]. However, the number of available columns is lower when focusing on state-of-the-art UHPSFC columns (i.e. columns packed with sub-2 μm fully porous particles or with sub-3 μm SPP). As mentioned in Section 3.1, because of the elevated mobile phase viscosity at the end of the unified chromatography gradient, the use of sub-2 μm fully porous particles was not adapted (system overpressure) and SFC-compatible columns packed with SPP particles of 2–3 μm represented the best compromise between backpressure and efficiency. Several stationary phase chemistries were selected, all having different and complementary properties (i.e. acidic and basic, neutral and charged, polar and hydrophobic ligands). Based on previous results [40] and on the poor availability, only seven stationary phase were selected for the analysis of 57 representative metabolites, including Sunshell Diol and 2-ethylpyridine (2-EP), Nucleoshell HILIC (zwiterionic), Poroshell HILIC (bare silica), Kinetex C18 Polar, Synergi Polar (phenyl ether) and Cosmosil 3-hydroxyphenyl (3-HP). The two last columns were packed with 2.5 μm fully porous particles and, thus, their kinetic performance should be slightly reduced compared to the other ones, packed with SPP particles.

To compare these stationary phases, a generic mobile phase was selected based on the literature and MS compatibility, including CO_2 and 10 mM ammonium formate dissolved in MeOH/water (95:5) as the co-solvent. A generic gradient ranging from 2 to 100% co-solvent in 7 min (including a 1 min isocratic step at 2% and a 1 min isocratic step at 100% co-solvent) was applied. The 57 selected compounds were analyzed under these unified chromatography conditions. Their peak shapes were visually inspected and divided into three groups, namely non-detected analytes, analytes presenting distorted peak shapes and analytes with satisfactory peak shapes, based on visual inspection of the chromatograms obtained. The corresponding proportions for the three groups on the seven columns were reported in Fig. 3. First, it appears that some compounds were not detected on the different columns (approximately 20% of the metabolites) and were almost the same regardless of the column chemistry. A few of the non-detected metabolites possessed one or several phosphate groups (i.e. adenosine di and triphosphate, ethanolamine phosphate, fructose 6-phosphate...) and some other ones contained carboxylic acid groups (i.e. citric acid, tartaric acid, picolinic acid...). The putative explanations for this loss of compounds were: i) a possible precipitation of these analytes in the starting conditions, due to poor solubility, ii) a too strong retention on the stationary phase, or iii) no ESI ionization or fragmentation in MS/MS. For the other metabolites, two columns clearly displayed a higher proportion of distorted peaks, namely the Synergi Polar and Kinetex C18 Polar columns, with 46 and 61%, respectively. This result was not surprising since these two columns are normally designed for reversed phase chromatography, and are not well suited to analyze polar metabolites. However, the Kinetex C18 Polar was the only one to separate two of the fatty acids present in the set of analytes (namely tricosanoic acid and oleic acid). On the six remaining columns, these two metabolites had the same retention time. This can be explained by the fact that these two fatty acids should interact only through their identical carboxylic group with a polar stationary phase and thus, no discrimination can be made. On the contrary, when using a stationary phase having hydrophobic selectivity, the difference in their carbon chain length can be used as a separation lever. This is an additional proof that hydrophobic selectivity exists in SFC [41]. For the five remaining columns, the results were quite comparable with about 50–60% of gaussian peaks (including some carbohydrates, amino acids, nucleosides, small organic acids, sulfated metabolites, quaternary ammonium and lipids) and 20–30% of distorted peaks. For the representative set of metabolites, the three best columns were the Poroshell HILIC (bare silica), followed by Nucleoshell HILIC (zwitterionic bonding) and Sunshell Diol (diol bonding). The analytes displaying distorted peaks and a few non-detected metabolites were further evaluated during the mobile phase optimization described in the following section.

The selectivity of the different stationary phase chemistries was also assessed. For this purpose, the retention times of the detected analytes on four representative columns were reported in Fig. 4.

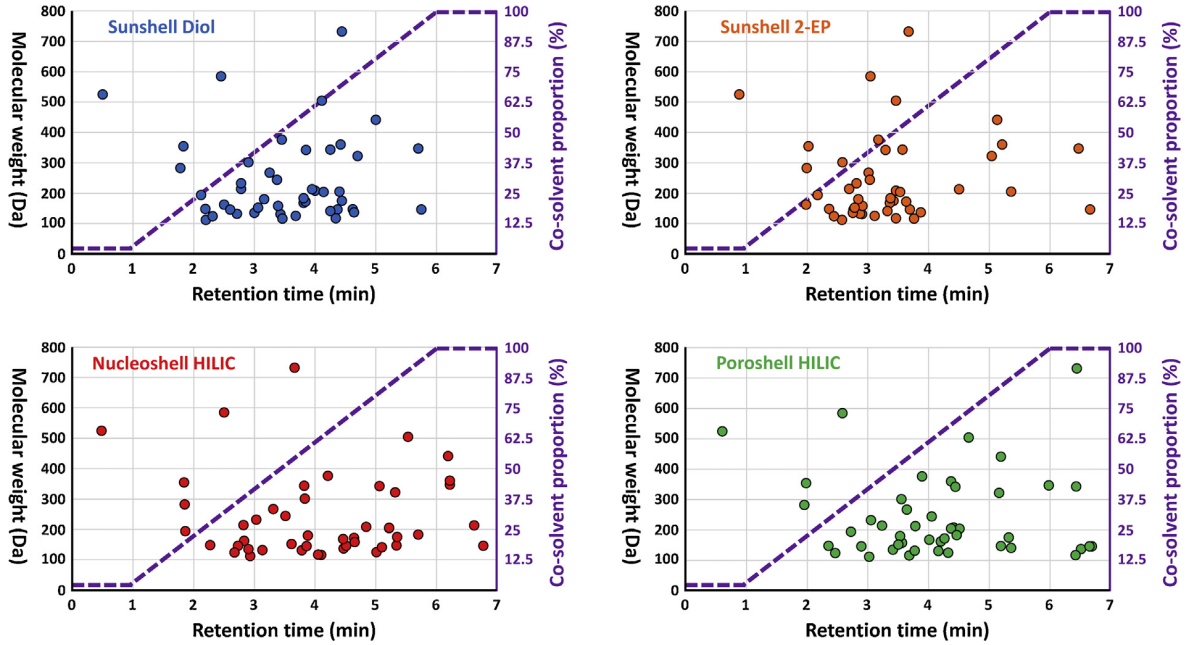


Fig. 4. 2D-maps representing the SFC (retention times) and MS (MW, Da) information for four representative stationary phases. The percentage of co-solvent in the gradient as a function of the analysis time was also represented using a dashed purple line.

This representation allowed distinguishing the column offering the best spreading of the compounds over the gradient. As an example, most of the metabolites were eluted within a short retention time window between 2 and 4 min on the Sunshell 2-EP. On the contrary, the repartition of the metabolites was better distributed along the gradient with the Poroshell HILIC and Nucleoshell HILIC. The Nucleoshell HILIC had a gradient coverage somehow in between the 2-EP and the two other columns.

In conclusion, two stationary phases can be used for the successful analysis of apolar and polar metabolites under unified chromatography gradient conditions, namely the Poroshell HILIC and Nucleoshell HILIC. When using one of these two columns, very different analytes such as amino acids, carbohydates, nucleosides, positively and negatively charged compounds, but also lipophilic compounds such as lipids and steroids, could be successfully analyzed using such conditions (see Fig. 5). When selecting an adequate injection solvent (water/acetonitrile 50:50) to avoid dissolution issues, it was even possible to simultaneously analyze one of the most lipophilic and one of the most hydrophilic compounds of the set of metabolites, while still getting sufficient retention for both analytes and acceptable peak shapes (see Fig. 6). This impressive chromatogram is the proof that SFC can be applied for the analysis of a very wide range of metabolites, which represents a great asset in the field of metabolomics.

3.4. Optimization of the mobile phase conditions

3.4.1. Selection of the optimal organic modifier

In SFC, the addition of an organic modifier to the mobile phase is crucial to elute the most polar compounds from the column. Methanol (MeOH), ethanol (EtOH), isopropanol (IprOH) or acetonitrile (ACN), are commonly used, with MeOH possessing the highest elution strength and ACN the lowest (lower hydrogen bonding properties compared to small alcohols). In some cases, some organic modifier mixtures can also be employed to tune the mobile phase elution strength [42], and improve peak shapes. Besides the organic solvent, 5% water was also systematically added to the mobile phase in this study, to elute the highest number of metabolites from the column with suitable peak shapes, and achieve excellent retention time repeatability.

Initially, different organic modifier (binary and ternary) mixtures were tested on the Poroshell HILIC. On the 57 tested metabolites, the best compromise was obtained with MeOH/Water 95/5.

3.4.2. Selection of the optimal salt concentration

Another important parameter that needed to be adjusted was the salt concentration in the mobile phase. Based on the literature, ammonium formate (AmF) appeared as the most promising one in SFC-MS, and was therefore selected as a starting point [43,44]. Generally, the most common salt concentrations are comprised between 5 and 10 mM. However, this translates into a maximum concentration of only 2–4 mM AmF in the mobile phase, when using 60% CO_2/40% organic modifier, and around 4–8 mM in 20% CO_2/80% organic modifier. Due to the extreme polarity and ionic character of most of our metabolites, a non-negligible number of peaks were broad and tailed with such low AmF proportion. Hence, higher concentrations of AmF (i.e., 20, 50, 100 and 150 mM) were also tested on the Poroshell HILIC column, to further improve peak shapes.

First, the salt concentration affected the retention of polar and ionic analytes. The use of concentrations of AmF equal or higher than 50 mM highlighted different patterns: for most of the acidic compounds, the retention increased with AmF concentration. Only a few acidic compounds (i.e. thymidine monophosphate, Fig. 7) showed a slight retention decrease. For zwitterionic (i.e. lauroylcarnitine) and basic species (i.e. lysine, Fig. 7) instead, the use of higher salt concentration always decreased retention. It is therefore clear that, by increasing the salt ionic strength, an ion pairing effect is increasingly seen. For acidic compounds, the pairing with ammonium ions resulted in a lower repulsion with the negatively charged silanolate groups. For basic and zwitterionic analytes, a reduction in the strong interactions with the silanolates on the stationary phase was observed, translating into a retention decrease.

Besides the variation of retention time with salt concentration, peak shapes were also affected. As shown in Fig. 7, two highly hydrophilic metabolites, namely thymidine monophosphate (neg-

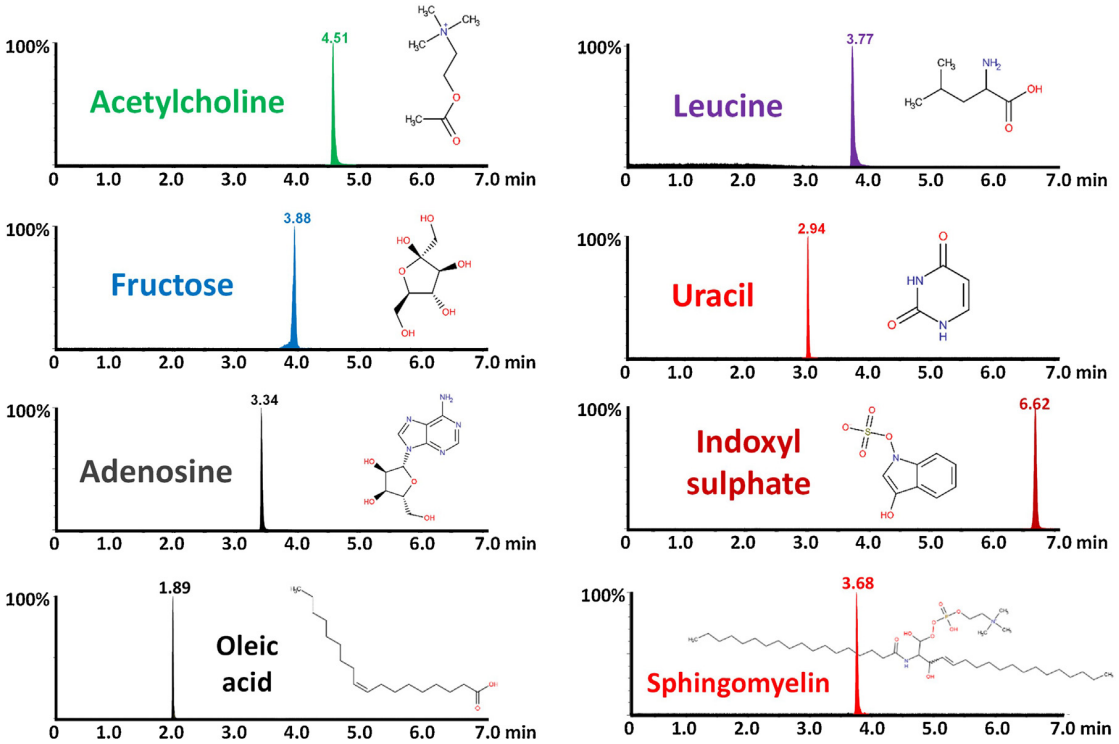


Fig. 5. Chromatograms obtained for several representative polar and apolar metabolites using unified chromatography gradient conditions. Mobile phase: CO_2 + 10 mM in MeOH/water (95:5). Column: Nucleoshell HILIC 2.7 μm, 100 × 3.0 mm. Mobile phase temperature of 40 °C and backpressure of 120 bar.

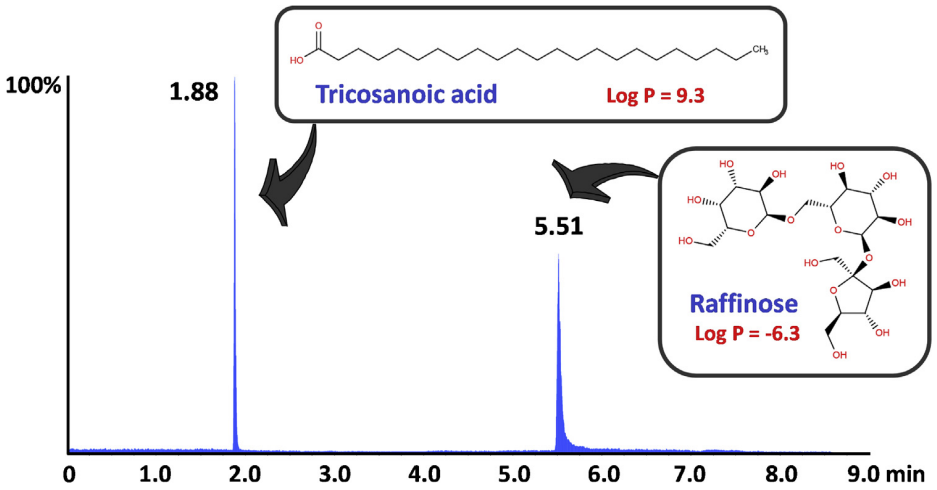


Fig. 6. Chromatogram obtained for the simultaneous injection of tricosanoic acid and raffinose, using acetonitrile/water (50:50) as sample diluent and unified chromatography gradient conditions. Mobile phase: CO_2 + 10 mM in MeOH/water (95:5). Column: Nucleoshell HILIC 2.7 μm, 100 × 3.0 mm. Mobile phase temperature of 40 °C and backpressure of 120 bar.

atively charged) and lysine (positively charged) showed important peak shape deformations at 10 mM AmF, which could be related to their potential precipitation in the mobile phase (in presence of a high proportion of CO_2). Therefore, increasing AmF to 150 mM greatly improved the solubility of these highly polar metabolites, leading to narrower and more symmetrical peaks.

3.4.3. Selection of the additive nature

The initial selection of AmF was given mainly by its popularity among the MS compatible additives available for SFC. Nevertheless, 150 mM is a high concentration which can induce solubility and sensitivity issues. It was, therefore, interesting to evaluate the use of other salts and their impact on the chromatographic separation, as well as on the quality of the MS spectra. One interesting additive is ammonium fluoride. In the literature, there are only a few papers reporting its use in LC–MS [45,46], to improve compounds ionization in ESI- conditions, mostly thanks to the high basicity of the fluoride, which can efficiently deprotonate acidic compounds [47]. To the best of our knowledge, ammonium fluoride was never used in SFC-MS.

The addition of 2.5 mM NH_4F to the organic modifier was evaluated in comparison to 150 mM AmF. Besides the expected increase in signal intensity in ESI- mode, some relevant chromatographic modifications were also noticed, such as the retention times (i.e., retention decreased and increased for acidic and basic compounds, respectively), and peak shapes (i.e. better peak shapes of thymi-

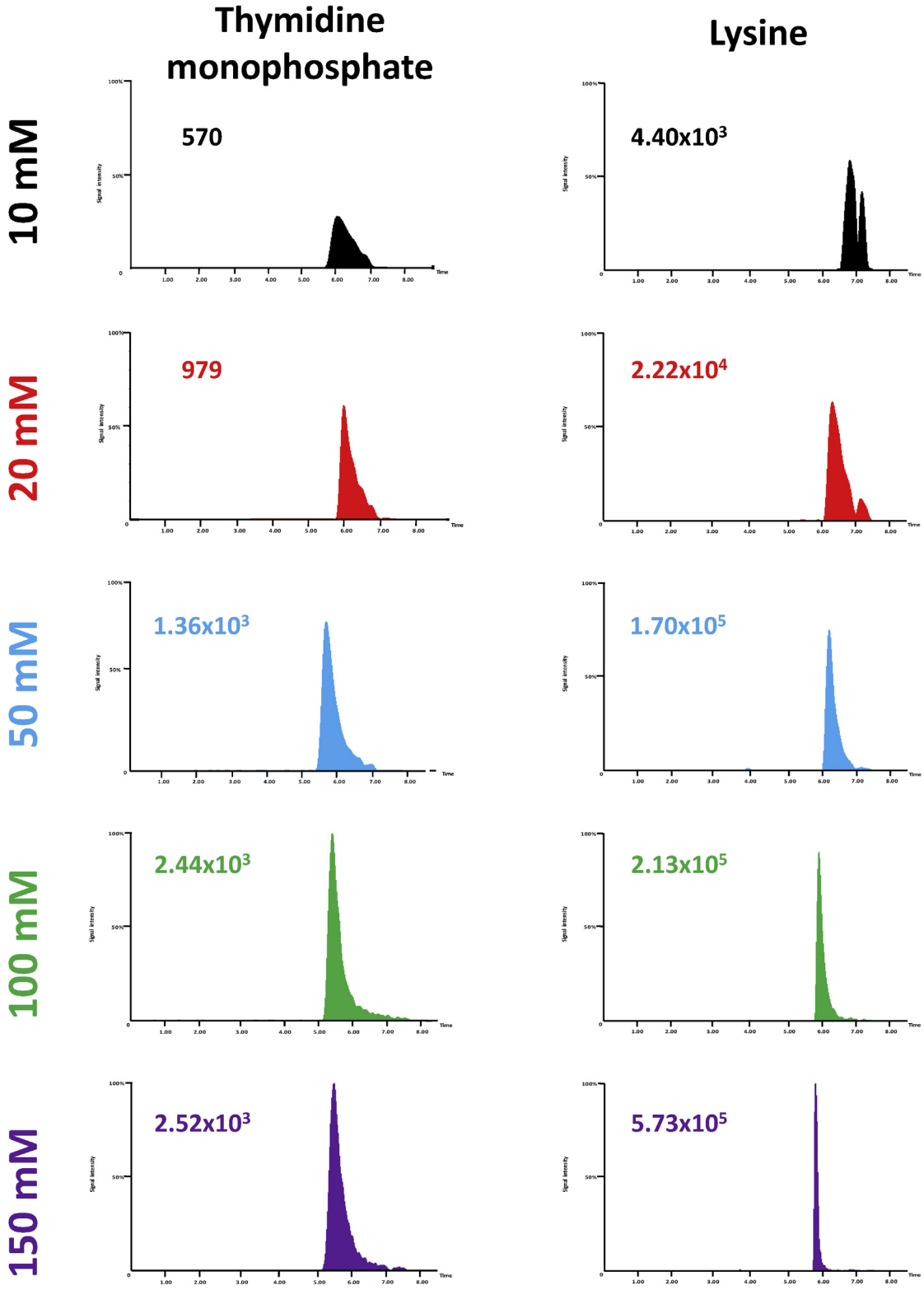


Fig. 7. Comparison of the chromatograms obtained at five different AmF concentrations: 10 mM (black), 20 mM (red), 50 mM (blue), 100 mM (green) and 150 mM (violet) for thymidine monophosphate (left) and lysine (right). Column: Agilent Poroshell 120 HILIC. Mobile phase: CO_2 – 95/5 MeOH/H_2O + AmF. Mobile phase temperature of 40 °C and backpressure of 120 bar. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

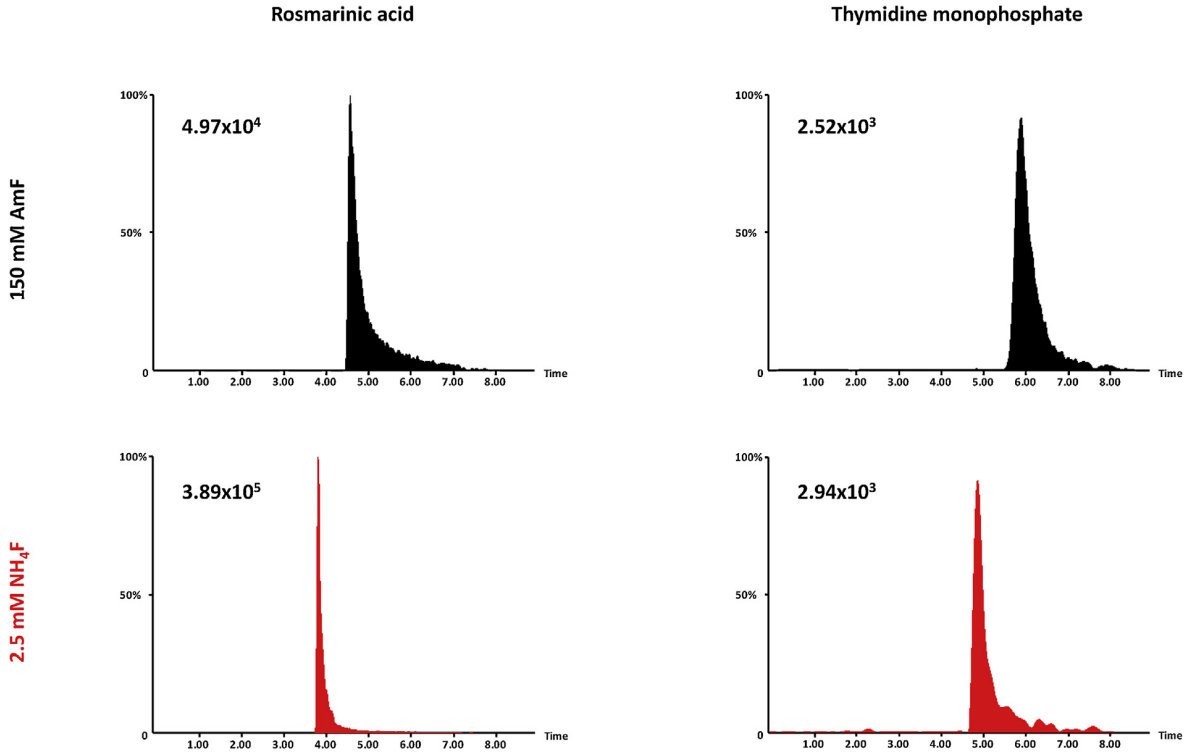


Fig. 8. Comparison of the chromatograms obtained with 150 mM AmF (black) and 2.5 mM NH_4F (red), for rosmarinic acid (left) and thymidine monophosphate (right). Column: Agilent Poroshell 120 HILIC. Mobile phase: CO_2 – 95/5 MeOH/H_2O + salt. Mobile phase temperature of 40 °C and backpressure of 120 bar. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

dine monophosphate and rosmarinic acid) when switching from 150 mM AmF to 2.5 mM NH_4F (see Fig. 8). The retention decrease for acidic compounds was mostly due to the ionic strength reduction when using ammonium fluoride, resulting in a much lower concentration of ammonium and, therefore, the repulsion of acidic substances with silanolates increased. For basic compounds, fluoride ions are present at low concentration, reducing the shielding of positively charged metabolites and, hence, enabling a better interaction with the stationary phase. The peak shape improvement observed for different metabolites (Fig. 8) can be explained by the fact that ammonium fluoride might provide a more acidic environment *vs.* ammonium formate. Therefore, there would be less ionized compounds suffering from the electrostatic repulsion with the stationary phase, thus enabling more symmetrical and narrower peaks. However, the main challenge associated with the use of ammonium fluoride is its solubility: although it is perfectly soluble in water, it possesses a low solubility in small alcohols such as methanol, and some precipitation issues were observed at a concentration of 2.5 mM. Therefore, it is advised to use a lower concentration of ammonium fluoride, such as 1 mM.

It was finally interesting to test whether the combination of ammonium formate and ammonium fluoride in the organic modifier could be even more powerful than each individual additive. A 95/5 MeOH/Water + 50 mM AmF + 1 mM NH_4F was then tested as organic modifier and compared to 50 mM AmF and 10 mM AmF. The results displayed in Fig. 9 clearly indicated that the combination of both salts allowed achieving the best possible peak shapes for some of the most problematic metabolites (i.e. histidinol, lysine, folic acid, rosmarinic acid, thymidine monophosphate and adenosine monophosphate). With this choice of additives in the mobile phase on the Poroshell HILIC, it was possible to increase the percentage of compounds with optimal peak shape and to decrease the compounds that were not detected with the initial buffer concentration, as shown in Fig. 10A. Most of the compounds that were still not detected possess one or more phosphoric acid groups, the exceptions are sodium citrate, for which it is believed that there was a solubility issue in the mobile phase, and picolinic acid, an analyte that might present a fragmentation issue in the ESI source (Fig. 10B).

4. Conclusion

The goal of this work was to evaluate the applicability of SFC-MS for the simultaneous analysis of polar and apolar metabolites, for a potential use in the field of metabolomics. For this purpose, a unified chromatography gradient from 2 to 100% organic modifier in CO_2 was systematically used to cover the widest possible range of analytes. Then, various column chemistries and dimensions as well as mobile phase conditions (organic solvent choice and composition, additives nature and concentration, temperature, etc.) were tested to find out the best combination. When taking into account the kinetic performances and upper pressure limit of our SFC system, it appears that the most appropriate stationary phase technology to work with unified gradient was the columns packed with sub-3 μm superficially porous particles, due to their high efficiency and the limited generated pressure. In terms of chemistry, the best stationary phases for this application were the Agilent Poroshell HILIC (bare silica) and Macherey-Nagel Nucleoshell HILIC (silica bonded with zwitterionic ligand). For the mobile phase, a significant amount of salts was necessary to i) increase solubility, ii) decrease retention and iii) obtain suitable peak shapes for ionizable species. Then, a mixture of 95% MeOH and 5% water, with 50 mM ammonium formate and 1 mM ammonium fluoride, as organic modifier was employed. In addition, these "special" conditions were applied to a representative set from the HMDB, containing 57 metabolites and covering a wide range of chemical

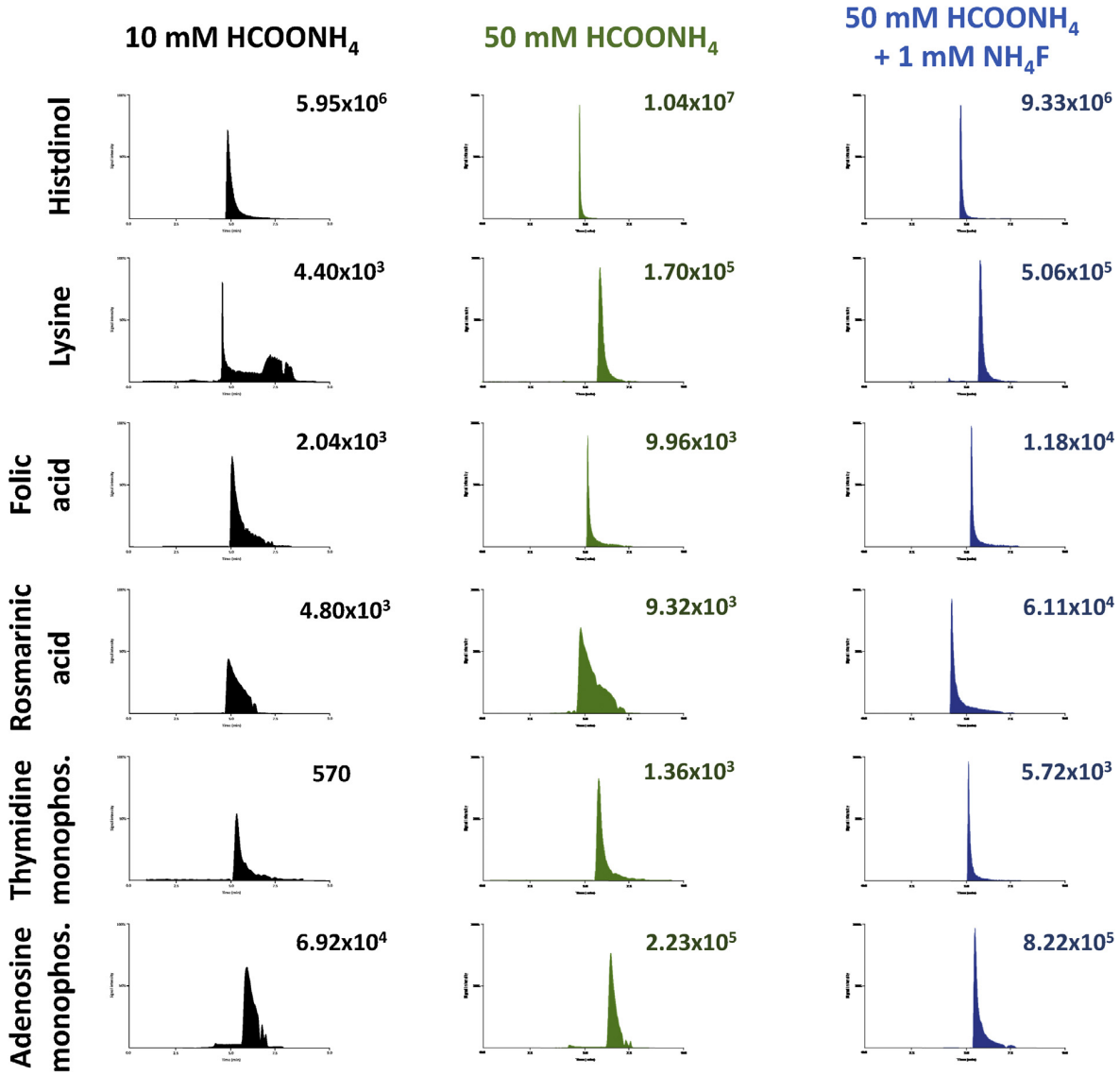
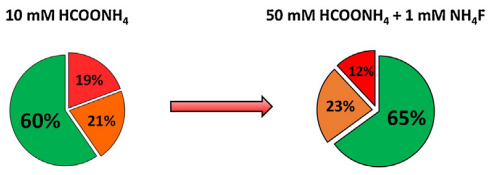


Fig. 9. Comparison of the chromatograms obtained with 10 mM AmF (black), 50 mM AmF (green) and 50 mM AmF + 1 mM NH_4F (blue). From the top to the bottom: histidinol, lysine, folic acid, rosmarinic acid, thymidine monophosphate, adenosine monophosphate. Column: Agilent Poroshell 120 HILIC. Mobile phase: CO_2 – 95/5 MeOH/H_2O + salt. Mobile phase temperature of 40 °C and backpressure of 120 bar. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

A)



B)

Compound name	Chemical nature
Phosphoserine	*Ester of serine and phosphoric acid*
Sodium citrate	*Salt of organic acid*
Fructose-6-phosphate	*Phosphate-containing fructose*
Adenosine diphosphate	*Nucleotide*
Picolinic acid	*Organic acid*
Inosine triphosphate	*Intermediate seen in ATP and GTP synthesis*
Phosphorylethanolamine	*Ethanolamine derivative with phosphate*

Fig. 10. A) Comparison of the proportions of non-detected peaks (red), distorted peaks (orange) and Gaussian peaks (green) obtained on the Agilent Poroshell 120 HILIC column using 10 mM AmF (left) and 50 mM AmF + 1 mM NH_4F (right) in the mobile phase. Mobile phase: CO_2 – 95/5 MeOH/H_2O + salt. Mobile phase temperature of 40 °C and backpressure of 120 bar. B) Table listing names and chemical nature of the compounds not detected with the optimized conditions, consisting of mobile phase CO_2 – 95/5 MeOH/H_2O + 50 mM AmF + 1 mM NH_4F on the Agilent Poroshell 120 HILIC. Mobile phase temperature of 40 °C and backpressure of 120 bar. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

classes, such as nucleosides, nucleotides, small organic acids, small bases, sulfated/sulfonated metabolites, poly-alcohols, lipid related substances, quaternary ammonium metabolites, phosphate-based substances, carbohydrates and amino acids. Finally, it was also demonstrated that fatty acids (highly lipophilic metabolites, with log P around 10) and carbohydrates (very hydrophilic metabolites, with log P below −5) were simultaneously analyzed using these generic conditions in SFC, with a sample diluent consisting of 50%ACN/50%water.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.chroma.2018.05.055.

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II.3.2 Analysis of a comprehensive library of metabolites and evaluation of biological matrices

This work is a follow-up of the article presented in section II.3.1. After developing and optimizing the UHPSFC method, the focus has been put in understanding which categories of endogenous metabolites can be successfully analyzed and which present some issues. To do so, the Sigma Metabolite Library, composed of 597 metabolites, was entirely assessed with a UHPSFC coupled to a HRMS system (QqTOF). Afterwards, the impact of two biological matrices, urine and plasma, on the overall quality of the developed UHPSFC method was taken into consideration, assessing the ME generated, as well as the quality of MS/MS spectra and, finally, the variability of the retention times for a library of 51 analytes spiked in both matrices.

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Applicability of Supercritical fluid chromatography–Mass spectrometry to metabolomics. II–Assessment of a comprehensive library of metabolites and evaluation of biological matrices

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ABSTRACT

In this work, the impact of biological matrices, such as plasma and urine, was evaluated under SFC–HRMS in the field of metabolomics. For this purpose, a representative set of 49 metabolites were selected. The assessment of the matrix effects (ME), the impact of biological fluids on the quality of MS/MS spectra and the robustness of the SFC–HRMS method were each taken into consideration. The results have highlighted a limited presence of ME in both plasma and urine, with 30% of the metabolites suffering from ME in plasma and 25% in urine, demonstrating a limited sensitivity loss in the presence of matrices. Subsequently, the MS/MS spectra evaluation was performed for further peak annotation. Their analyses have highlighted three different scenarios: 63% of the tested metabolites did not suffer from any interference regardless of the matrix; 21% were negatively impacted in only one matrix and the remaining 16% showed the presence of matrix-belonging compounds interfering in both urine and plasma. Finally, the assessment of retention times stability in the biological samples, has brought into evidence a remarkable robustness of the SFC–HRMS method. Average RSD (%) values of retention times for spiked metabolites were equal or below 0.5%, in the two biological fluids over a period of three weeks.

In the second part of the work, the evaluation of the Sigma Mass Spectrometry Metabolite Library of Standards containing 597 metabolites, under SFC–HRMS conditions was performed. A total detectability of the commercial library up to 66% was reached. Among the families of detected metabolites, large percentages were met for some of them. Highly polar metabolites such as amino acids (87%), nucleosides (85%) and carbohydrates (71%) have demonstrated important success rates, equally for hydrophobic analytes such as steroids (78%) and lipids (71%). On the negative side, very poor performance was found for phosphorylated metabolites, namely phosphate-containing compounds (14%) and nucleotides (31%).

1. Introduction

Due to the incredible heterogeneity of all the metabolites present in the human body, it has been quite difficult so far to develop generic analytical techniques for their determination [1–4]. Nonetheless, several efforts have been made with this aim, which mostly involve the use of ultra-high-performance liquid chromatography (UHPLC) [5–7] and high-resolution mass spectrometers (HRMS), such as the Orbitrap or QqTOF devices [8–10].

Despite all these achievements, there is still a lot of work to do in developing more comprehensive techniques, which can more efficiently analyze different categories of metabolites with contrasting chemical properties, going from lipids and steroids to amino acids and sugars. Recently the implementation of ultra-high performance super- or subcritical fluid chromatography (UHPSFC) [11] was assessed in the field of metabolomics, as an alternative technique which could be used instead of reversed-phase liquid chromatography (RPLC) or hydrophilic interaction chromatography (HILIC). In this paper [11], using a limited set of metabolites, it

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was successfully demonstrated how UHPSFC, coupled to a tandem MS system, was able to detect extremely different analytes such as lipids, nucleosides, sugars, small organic acids and so on within a single analysis on the same device. There are, however, several points that still need to be addressed. For example, it is important to assess the effect of different biological matrices on the quality and robustness of the developed UHPSFC method, with a special focus on the matrix effects being generated. Moreover, the number of metabolites previously used is rather limited compared to the real scenario in metabolomics. As an example, the Human Metabolome Database (HMDB) has registered around 110,000 metabolites in its database, and around 30,000 human metabolic and disease pathways are present in the Small Molecule Pathway Database (SMPDB) [12–15]. Considering this impressive number of potential compounds and targets, there is a strong need to increase the number of metabolites that must be tested under SFC conditions to check their detectability with this technique.

The aim of this study was therefore to assess the applicability of SFC, coupled to a high-resolution mass spectrometer, in the field of metabolomics by using an extended set of metabolites. The Sigma Mass Spectrometry Metabolite Library of Standards (MSMLS), composed of nearly 600 metabolites, has been employed to assess the detectability of these compounds under SFC–HRMS conditions. Moreover, urine and plasma samples spiked with a limited set of about 50 representative metabolites have been also evaluated under such conditions, to assess the impact of matrix effect (ME) on the intensity and the retention time variability of the tested compounds. Finally, the MS/MS spectra of this limited set of metabolites were analyzed in such matrices to check for possible interferences from the matrix components.

2. Materials and methods

2.1. Chemicals and reagents

The Sigma Mass Spectrometry Metabolite Library of Standards (MSMLS), composed of 634 pure standards (597 univocal analytes), including 37 quality control duplicates, was purchased from Sigma-Aldrich (Buchs, Switzerland). The 49 metabolites (Table S1), chosen among the 57 previously used in the first part of this study were purchased as standards from Sigma-Aldrich. Their description can be found in [11]. Methanol (MeOH) of OPTIMA LC/MS grade and water of UHPLC grade were purchased from Fisher Scientific (Loughborough, UK). Dichloromethane of puriss. p.a. grade (>99.9%), ammonium formate (AmF) of LC-MS grade and ammonium fluoride (NH_4F - >99.9%) were purchased from Sigma-Aldrich. Pressurized carbon dioxide (CO2) 4.5 grade (99.995%) was purchased from PanGas (Dagmerstellen, Switzerland).

2.2. Standard solutions preparation

The set of 49 metabolites used in the first part of this work were divided into six mother solutions, at a concentration of 500 µg/mL in ACN/H_2O 50/50 v/v. From these mother solutions, a dilution to 50 µg/mL in ACN/H_2O 50:50 v/v was then performed to obtain the standard solutions used for the analyses.

The Sigma MSMLS library is composed of seven 96-well plates. Once the 37 quality control duplicates have been removed, the remaining 597 metabolites were used to prepare stock solutions at 25 µg/mL, using different sample diluents as detailed in [16]. Dichloromethane was successively used as the sample diluent for hydrophobic analytes. Once the addition of solvent was made, each well plate was left agitating on a Thermomixer (Vaudaux – Eppendorf AG, Switzerland) for a total of 45 min at 900 rpm at room temperature. From the stock solutions at 25 µg/mL, final dilutions of each metabolite at 8 µg/mL were made with a mixture of ACN/water 50/50 v/v.

2.3. Biological samples and sample treatment

Urine samples were prepared according to a "dilute-and-shoot" protocol: six urines from healthy donors (3 males – 3 females) were centrifuged at 3000 × g for 6 min, then the supernatant was collected and filtered through a 0.45 µm nylon membrane. The filtered pooled urine was then divided into six aliquots, each of 250 µL as volume, each spiked with an aliquot from the six mother solutions previously described (500 µg/mL in H_2O:ACN 50:50 v/v), containing the set of 49 metabolites. The spiked urine aliquots have been further diluted up to 1000 µL with H_2O:ACN 25:75 v/v. Triplicate samples have been prepared. Final concentrations of analytes were 50 µg/mL. Urine was therefore diluted by a factor of 1:4. Samples were stored at −22 °C and thawed prior to injection.

Plasma samples were prepared following a "protein precipitation" pre-treatment: six different heparinized plasma samples, obtained from healthy donors, have been mixed to make a pool of plasma. PP_{ACN} was carried on this pool, by adding 9 mL of pure ACN to 4.5 mL of pooled plasma (dilution factor 1:2); the precipitated plasma was then centrifuged at 3000 X g for 6 min. The supernatant was collected and aliquoted six times creating aliquots of 250 µL each. Each aliquot was spiked with the six mother solutions already used for urine samples at a final concentration of 50 µg/mL and a final volume of 1000 µL. Samples were stored at −22 °C and thawed prior to injection.

2.4. UHPSFC–HRMS instrumentation and data treatment

All experiments were performed on a Waters Acquity UPC^2 system (Waters, Milford, MA, USA) equipped with a Binary Solvent Manager delivery pump, a Sample Manager autosampler which included a 10 µL loop for partial loop injection, a column oven and a two-step (active and passive) backpressure regulator (BPR). Acetonitrile and a mixture of MeOH/H2O 50/50 were used as the weak and strong wash solvents, respectively, with volumes of 600 µL and 200 µL. The chromatographic system was hyphenated to a Waters Xevo QqTOF via a double-T splitter interface from Waters [17]. Additional make-up solvent for SFC-MS operation was brought to the system by a Waters Isocratic Solvent Manager (ISM) pump, delivering pure MeOH at 0.3 mL/min. Empower 3.0 was used for the chromatographic system control.

The Waters Xevo QqTOF detector was operated in both positive and negative electrospray ionization (ESI) modes. Different parameters were optimized to obtain the highest sensitivity: source temperature at 150 °C, desolvation temperature at 450 °C, capillary voltage at ±2.5 kV. Nitrogen was used as a desolvation gas at 900 L/h. The cone voltage was fixed at 30 V. Acquisitions were performed in the *m/z* range of 50–1000 with a 0.25 s scan time. The instrument was periodically calibrated using the charged ions produced by a 0.5 mM sodium formate solution in acetonitrile/water 80/20 v/v. MassLynx 4.1 software was used for MS instrument control, data acquisition and data treatment. An analogic connection was established between the chromatographic system and mass spectrometer.

Chromatographic conditions were as following: the Poroshell HILIC 100×3.0 mm – 2.7 µm (Agilent, Santa Clara, CA, USA) was employed as the stationary phase, while the mobile phase was a mixture of CO_2 and MeOH/H_2O 95/5 v/v + 50 mM ammonium formate and 1 mM of ammonium fluoride. When analyzing biological samples, a Zorbax RX-SIL analytical guard column from Agilent (12.5 × 4.6 mm–5.0 µm) was fixed before the column, mounted on a guard column hardware kit high pressure from Agilent. Gradient mode was employed during all the analyses, more details can

be found in the first article of this series [11]. Backpressure was maintained constant at 105 bar, while mobile phase temperature was kept at 40 °C. The flow-rate was fixed at 0.9 mL/min. Injection volume was 3.0 µL.

To calculate RSD (%), as an estimate of metabolites retention times variability in biological samples, retention times were recorded and *inter-week* RSD (%) was calculated over a period of three weeks. Calculations were made with Microsoft Excel 2016. RSD values for each metabolite can be found in Table S1. The calculated RSD values were plotted as violin plots. Violin plots were created using Plotly Chart Studio (https://chart-studio.plot.ly) A more detailed description of their interpretation can be found in [18]. .

2.5. Estimation of the matrix effect

ME values were obtained following the Matuszewski's approach [19] and calculated by using the following Eq. (1):

$$ME(\%) = \frac{Peak\ area\ of\ post\ extraction\ spiked\ sample}{Peak\ area\ of\ standard\ in\ neat\ solution} \times 100 \quad (1)$$

An average of the peak areas' values of post extraction spiked samples obtained from three replicates and an average of the peak areas of neat standards was made. Matrix effect in the range between 50% ≤ ME ≥ 150% was labeled as "limited ME". ME values above 150% were considered as "Ion Enhancement", while values of ME below 50% were classified as "Ion Suppression". The ME values obtained for each metabolite can be found in the Supplementary Table S1.

3. Results and discussion

3.1. Matrix effect evaluation

The assessment of the ME generated at the electrospray ionization source is quite important, as it can give different considerations on the quality of the MS signals obtained. Moreover, its evaluation becomes essential as it can heavily influence the sensitivity of the analytical method for one metabolite, whose detectability might become hard to perform. The interfering compounds generating ME can be quite different and are strictly related to the type of matrix being employed. In the case of urine, as an example, such ME-generating elements are highly polar compounds with low molecular weights. For plasma, in addition to small polar molecules, there are also some lipophilic species such as phospholipids and triglycerides that can be responsible for ME. All these different components will affect the MS signal obtained, including both its intensity and fragmentation profile. Matrix effect can consist mainly of either ion suppression, that is a decrease in the MS signal intensity, or ion enhancement, where the MS signal intensity is higher than expected. To assess the performance of UHPSFC–HRMS with biological matrices, urine and plasma samples spiked with the set of 49 metabolites were evaluated following the Matuszewski's approach. Peak shapes of the used metabolites were symmetrical in most cases, with few cases of peak distortions (Fig. S1). Simple and generic sample treatment procedures (dilute and shoot for urine and protein precipitation for plasma) have been selected to mimic the most conventional workflow usually employed in untargeted metabolomics. More specific sample-treatment strategies, such as solid phase extraction (SPE) or solid liquid extraction (SLE), were not considered as they are known to be selective approaches, more suited for targeted analyses. In Fig. 1A and B, the average ME values generated by the detected metabolites were plotted as a function of their average retention times. In these two graphical representations, no relationship was found between the average ME value and the average retention of each analyte. This result points out how it is difficult to predict the ME effect for one given metabolite. Despite that, it is possible to detect some global trends related to the type of biological matrix employed. This is illustrated in Fig. 1C and D, where the average ME values have been classified in three categories: limited ME (50% ≤ ME ≥ 150%), ion suppression (50% ≤ ME) and ion enhance-

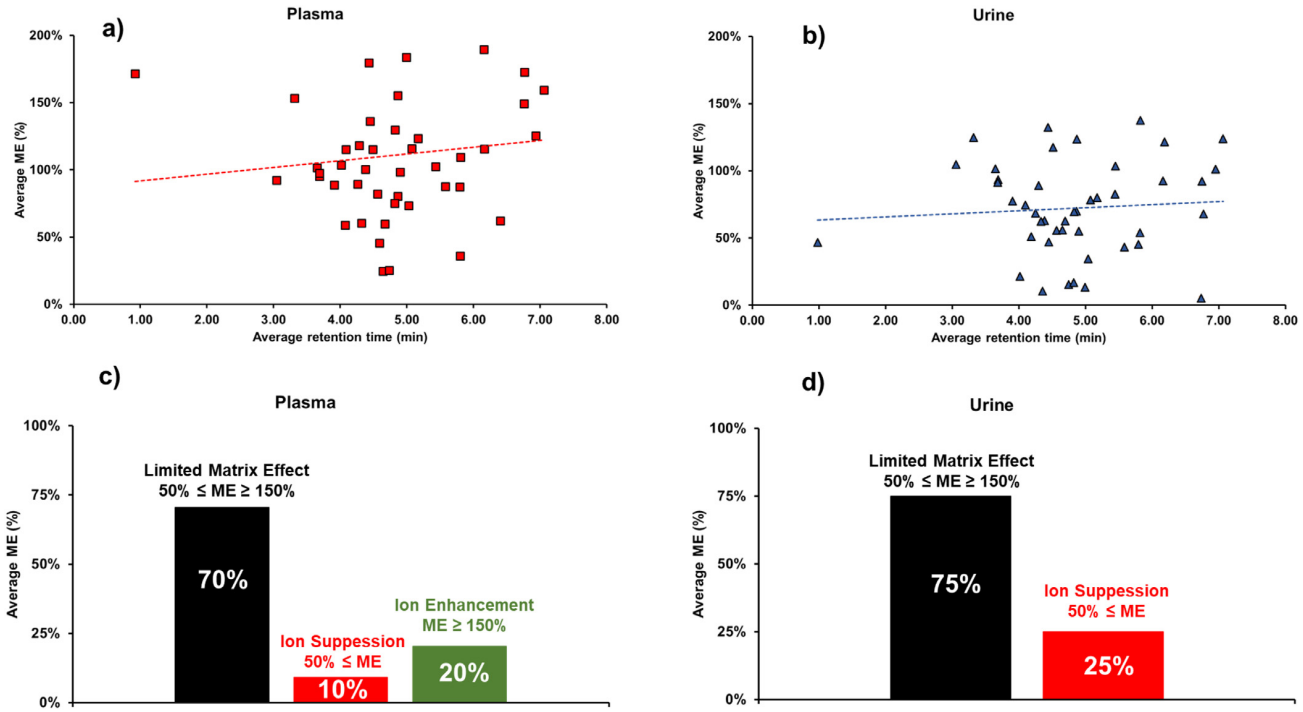

Fig. 1. (A) Scatter plot of the average matrix effect (%) as a function of the average retention time (min) for each metabolite in plasma. (B) Scatter plot of the average matrix effect (%) as a function of the average retention time (min) for each metabolite in urine. (C) Bar graph showing the distribution of the 49 metabolites according to the average matrix effect found in plasma. (D) Bar graph showing the distribution of the 49 metabolites according to the average matrix effect found in urine.

Table 1
List of metabolites showing a different behaviour of their matrix effect in urine and plasma.

	ESI ionization	Average Rt (min) URINE	Average ME (%) URINE	Average Rt (min) PLASMA	Average ME (%) PLASMA
Rosmarinic acid	NEG	4.86	124	4.88	155
Phosphorylethanolamine	POS	4.99	13	5.00	183
Picolinic acid	POS	5.44	104	5.40	345
Lysine	POS	4.44	132	4.45	179
Acetylcholine	POS	7.05	124	7.07	159
Adenosine monophosphate	POS	6.16	93	6.18	189
Caffeine	POS	3.32	125	3.32	153
Lauroylcarnitine	POS	6.76	68	6.77	173
Sphinganine	POS	4.00	21	4.02	103
Retinyl palmitate	POS	0.97	47	0.93	171

ment (ME $\geq$ 150%). Both biological matrices exhibited an overall limited ME influence in the ionization process for each detected metabolite (70% and 75% of metabolites in plasma and urine, respectively). However, different behaviors have been witnessed for ion suppression and enhancement. While in urine the remaining 25% of detected metabolites have all suffered from ion suppression, there was a predominance of ion enhancement (20% of detected metabolites) over ion suppression (10% of detected metabolites) with plasma matrix. Such differences in the ME behavior between these two matrices have already been witnessed in modern SFC-MS in the work of Desfontaine et al. [20]. In this paper, the authors have assessed the ME generated under UHPSFC-MS/MS conditions using a set of three UHPSFC stationary phases and have demonstrated that the ME seems to be mostly dependent from the choice of the stationary phase, rather than that of the sample treatment procedure (generic vs. selective). By taking into consideration the column chemistry being employed in this work (Poroshell HILIC – underivatized silica), the stationary phase is highly polar due to the presence of free silanols. As can be deducted from the cited work, with simple and generic sample treatment techniques such as the ones used in the present work, the use of polar stationary phases is associated with a predominance of ion suppression over ion enhancement for urine samples treated with the DS approach. The opposite scenario is observed when using plasma treated with the PP procedure. A further proof demonstrating the differences in ME behavior is that most of the compounds suffering from ion enhancement in plasma are, on the other hand, experiencing ion suppression (ME $\leq$ 50%) or limited matrix effect (50% $\leq$ ME $\geq$ 150%) in urine (Table 1). This behavior is almost exclusively present in ESI positive mode. A possible explanation of this phenomenon can be obtained by assessing the species present in each biological matrix, their elution times and their signal intensities. Fig. 2A and B are a representation of the different matrix-related species observed under ESI positive mode using the generic UHPSFC-MS method. As illustrated, there was an important discrepancy in the profile of the endogenous compounds present in each matrix. Indeed, plasma possesses a much more heterogeneous and more widespread profile, but with a few species generating high signal intensities. On the other hand, there is a lower variability of such ME-generating molecules in urine, but they were more intense. The situation in ESI negative mode was quite different, with a much lower number of matrix-belonging compounds observed with both matrices (Supplementary figures S2A and S2B). In ESI positive mode, Fig. 2A and B illustrate the difference in the complexity of these matrices: while for urine there are mostly small polar compounds such as urea, creatinine and inorganic ions, in plasma there are

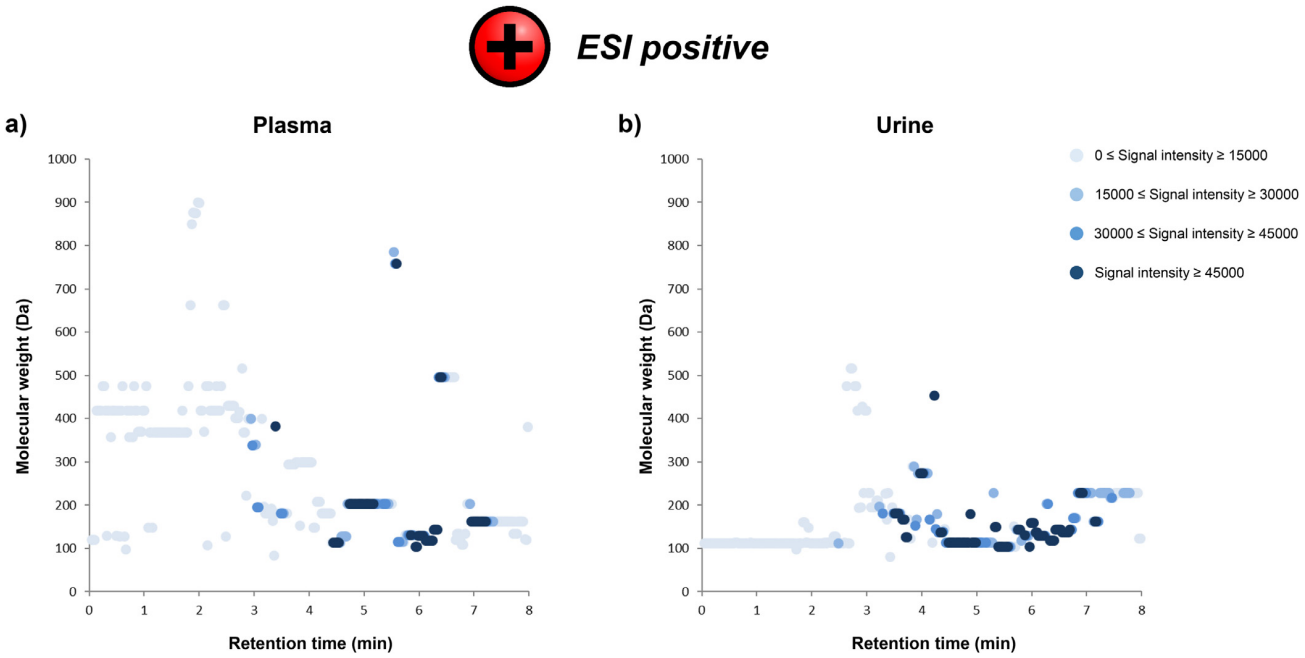

Fig. 2. (A) Ion map showing each compound belonging to the biological matrix assessed (plasma), according to their molecular weight (Da) and retention time (min). The signals with a more intense colour represent a higher signal intensity. (B) Ion map showing each compound belonging to the biological matrix assessed (urine) according to their molecular weight (Da) and retention time (min). The signals with a more intense colour represent a higher signal intensity.

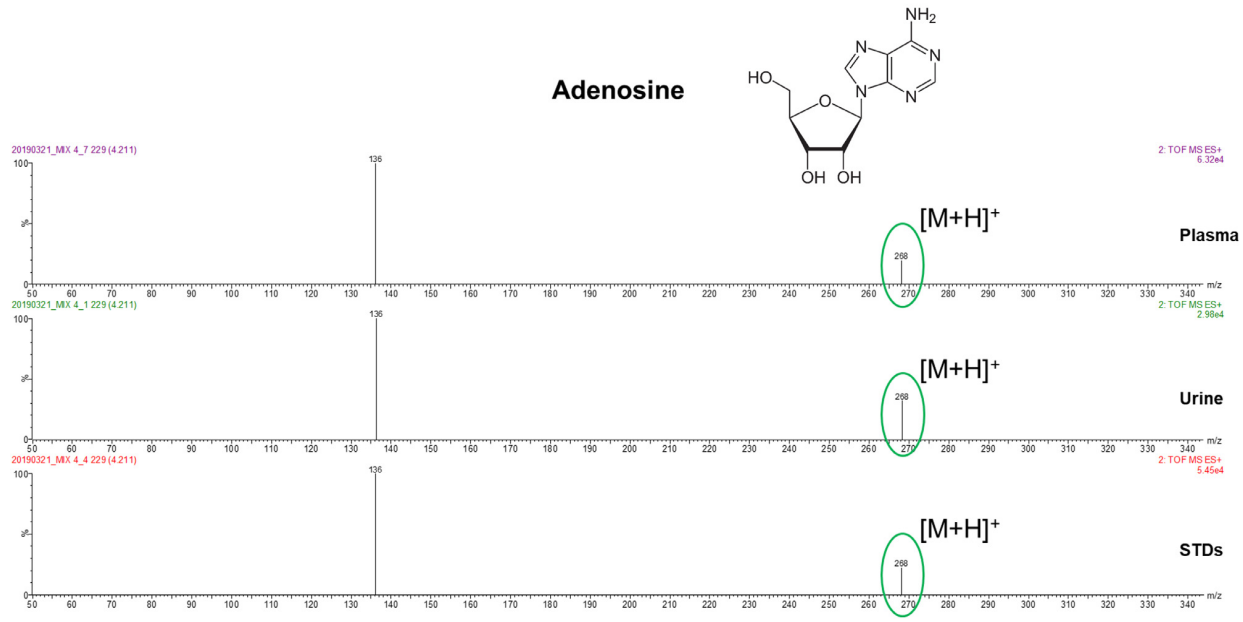

Fig. 3. MS/MS spectra for adenosine in plasma (upper signal), urine (middle signal) and in neat standard solution (lower signal).

also more hydrophobic components such as phospholipids and fat-soluble vitamins. This higher diversity could explain the insurgence of a more variegated ME profile, as witnessed in Fig. 1.

A final point that was assessed revolved around the possible presence of metal ion clusters in SFC-MS [21,22]. In their articles, the authors have witnessed and described an important contribution of ion suppression originating from the presence of metal ions in biological matrices, generating therefore metal ion clusters which greatly impact the signal intensities of those analytes coeluting with the inorganic ions. Their presence was, therefore, assessed in this work but no manifestation of such clusters was found. This could be surely dependent from the different MS systems being used, in the type of ESI ionization source employed and, finally, by differences in the sample preparation stage.

3.2. MS/MS spectra evaluation

In the field of metabolomics MS/MS fragmentation patterns are commonly used in the annotation and identification of signals. Therefore, the ability of high-resolution MS instruments to perform tandem MS/MS analyses, and to subsequently generate MS/MS spectra, is of primary importance in the metabolomic workflow. This is even more relevant when assessing real-life samples as there is a preponderant presence of endogenous contaminants specific for a given biological matrix, which could hamper the quality of MS/MS spectra. In a similar way to the ME during the ionization phase, these matrix-belonging species can also cause some issues during the stage of ion fragmentation, since some of them co-elute with the metabolites of interest. Therefore, the quality of the MS/MS spectra generated in UHPSFC mode was also assessed by comparing the MS/MS profiles of the analytes as standards vs. those spiked in treated urine and plasma samples. Fig. 3 shows an illustrative example of how the presence of the biological fluid did not affect the MS/MS spectra profile. For adenosine, as example, no interferences were recorded with any biological matrix. On the other hand, Fig. 4 depicts another illustrative case in which the selected metabolite (i.e. xanthurenic acid) is subjected to a selective influence of endogenous compounds related to the type of matrix being analyzed. No interferences were observed with urine, and the MS/MS spectra were identical to those obtained with the standard. Additionally, the presence of a $[M + H]^+$ at *m/z* of 184 was observed in the plasma sample. This ion comes from the dissociation of glycerophosphocholines, a component widely present in total plasma phospholipids population, into trimethylammonium-ethyl phosphate ions, as already reported [23]. Finally, Fig. 5 shows a third illustrative example with a different behavior. Here the MS/MS spectra of trigonelline presented always some interferences, whatever the biological fluid (plasma and urine). Furthermore, it is important to notice that such interferences are more common when employing the MS instrument in the ESI positive mode. In ESI negative mode, the significant lower presence of such matrix components, as previously discussed in Figures S2A and S2B, translates into a lower probability of interferences when generating MS/MS spectra.

Once these three behaviors were identified, the MS/MS spectra generated by the entire set of 49 metabolites were assessed. 63% of the compounds were characterized by an absence of interferences in any biological matrices. Out of the remaining 37%, 21% suffer from interferences in only one matrix, and 16% in both matrices. In Fig. S3 the percentages found for each ESI modality have been reported. As previously indicated, an important impact originating from the presence of the biological matrix was observed in ESI positive, while the number of components associated with urine and plasma is much lower in ESI negative. Therefore, the MS/MS spectra in ESI negative mode will always contain less interferences from matrix components.

3.3. Assessment of retention times stability

Once having assessed the influence of the matrix on the metabolites in the ionization process and MS/MS fragmentation profile in UHPSFC, another important aspect that must be evaluated is the variability of retention times when employing biological matrices treated with simple and generic sample treatment processes. This point is relevant since retention times must be used, along with other parameters, for the annotation and formal identification of metabolites obtained in untargeted acquisition. The reference chromatographic technique used in metabolomics is ultra-

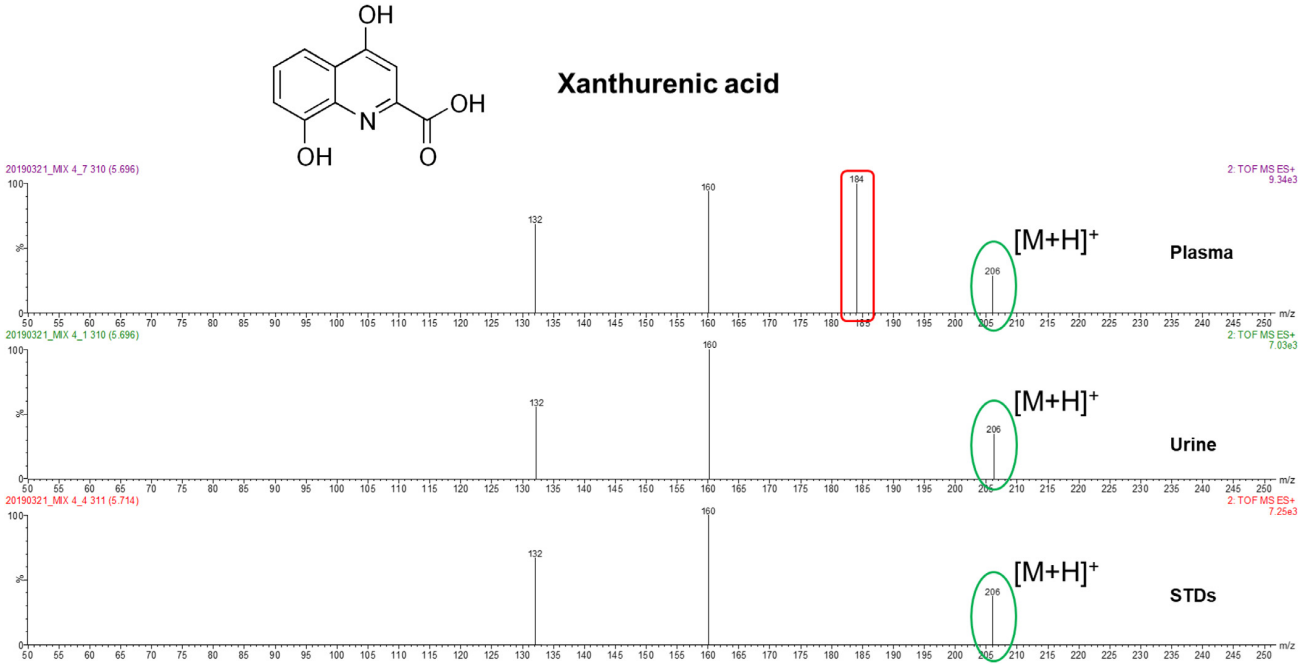

Fig. 4. MS/MS spectra for xanthurenic acid in plasma (upper signal), urine (middle signal) and in neat standard solution (lower signal).

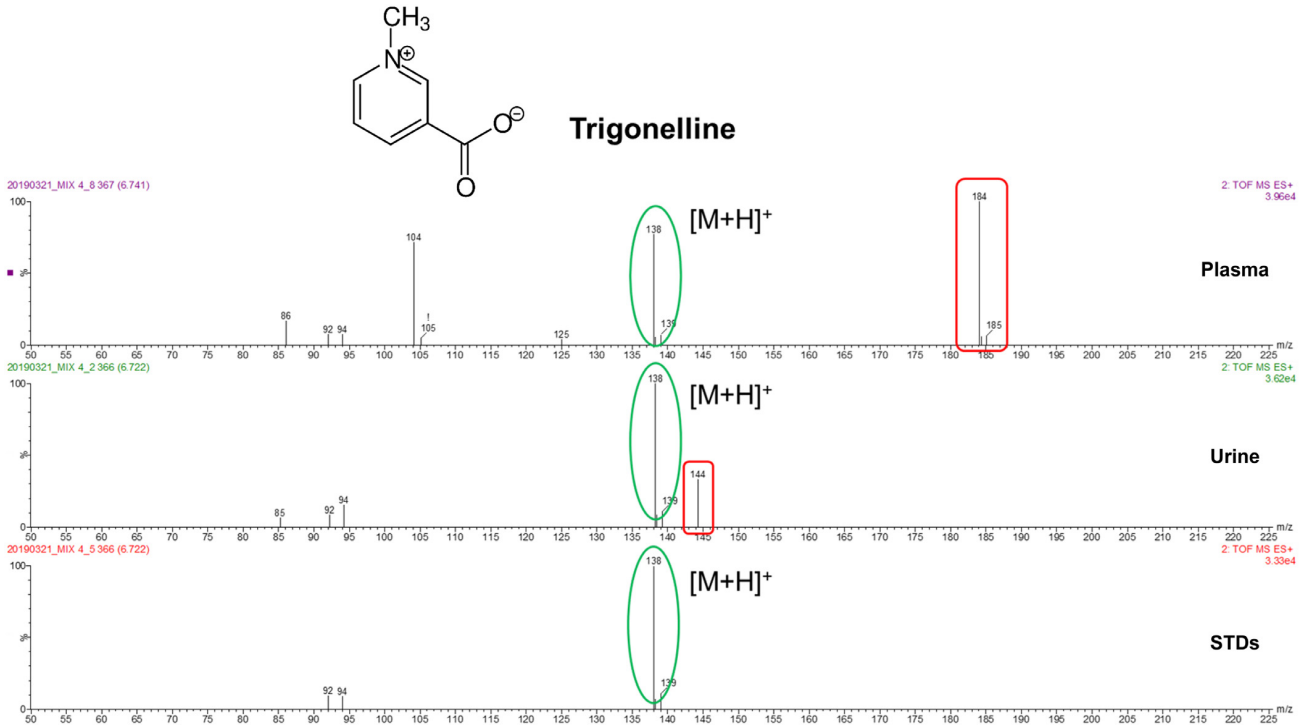

Fig. 5. MS/MS spectra for trigonelline in plasma (upper signal), urine (middle signal) and in neat standard solution (lower signal).

high performance liquid chromatography (UHPLC), which is known to possess a high degree of robustness and repeatability when using reversed-phase column under various analytical conditions, even in presence of biological matrices. The robustness and repeatability of SFC has however been scarcely explored. While the old generation instruments were not able to properly handle the super- / subcritical mobile phase and ensuring high repeatability, this issue has been recently resolved with the introduction of modern UHPSFC systems. The latter have become very robust [24,25] and demonstrated an excellent repeatability of retention times with standards and biological matrices as demonstrated in [18]. Sample preparation procedures used in untargeted metabolomics are commonly minimal to reduce the losses of analytes present at very low concentrations and to increase the coverage yield of the metabolome. However, it also means that more interfering compounds from the matrices will be regularly injected into the chro-

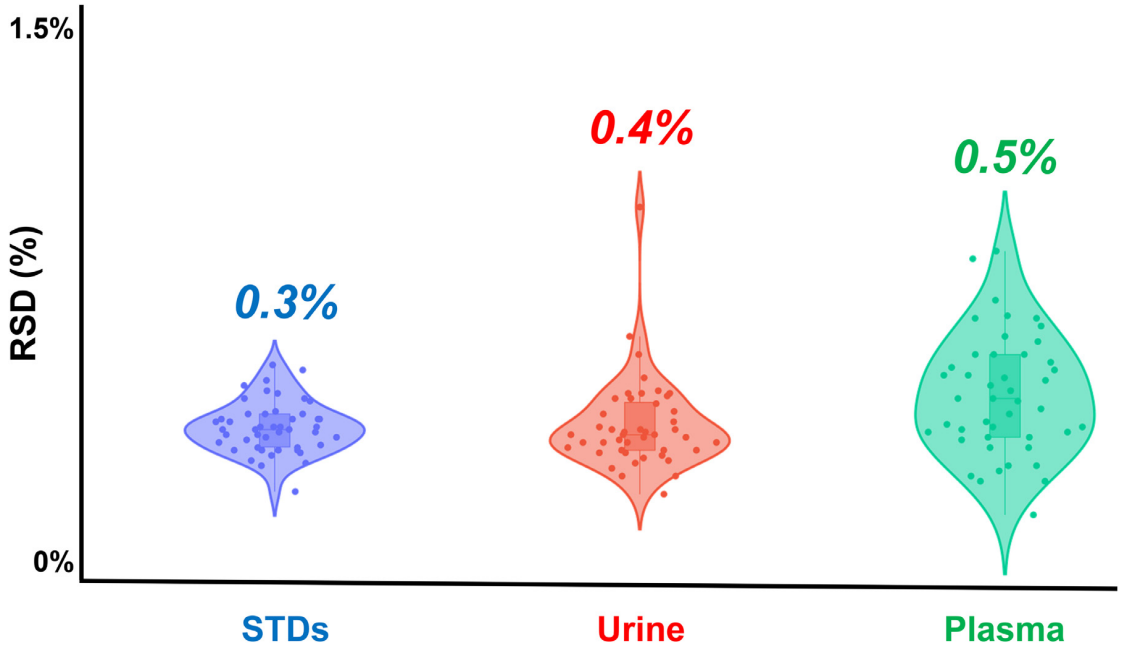

Fig. 6. Violin plots representing the population of RSD (%) values calculated for the 49 metabolites in neat standard solutions (blue), urine (red) and plasma (green). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

matographic system, which could remain retained by the stationary phase and poorly eluted. Therefore, it was important to assess whether the developed UHPSFC method can still generate an acceptable retention time repeatability. For that purpose, the retention times of the 49 metabolites were recorded over a period of three weeks, and a relative standard deviation (RSD%) was calculated for each matrix and for standards. The data was then represented using violin plots to easily visualize and compare RSD obtained from standards, urine and plasma samples (Fig. 6). Average RSD (%) values were extremely low: standards generated an average RSD of 0.3% over the three weeks, while urine (average RSD of 0.4%) and plasma (average RSD of 0.5%) did not highlight dramatic changes in the retention profile repeatability. Metabolites in plasma showed a slightly higher variability compared to those in urine, as the more elongated shape of the violin plot observed for plasma clearly indicates that there are more analytes generating higher RSDs than in urine. This trend might arise because of the higher number of matrix-related endogenous compounds present in plasma over urine, as already discussed (Fig. 2). Nonetheless, the very low variabilities found in all biological matrices is another support for the claim that UHPSFC has reached a very similar performance level to UHPLC. The excellent results obtained here are mostly due to the presence of a limited proportion of water in the mobile phase, which is known to improve repeatability in UHPSFC, as demonstrated in [20].

3.4. Analysis of the sigma MSMLS under UHPSFC–HRMS conditions

The next step was to increase the number of metabolites tested under the developed conditions above the panel of 49 compounds used so far. For this purpose, the Sigma Metabolite Library of Standards (MSMLS) was evaluated. Its variety and diversity of the species contained represents an interesting benchmark to further demonstrate the applicability of a novel analytical technique, such as UHPSFC–HRMS, in metabolomics. The entire library was therefore screened using the already optimized conditions and a detection rate of 66% was reached under the developed conditions. In Fig. 7, the detection percentages for each class of compounds are represented on a spider graph. Several interesting trends can be described. First of all, high success rates were found for some categories, which are generally not well detected with classical UHPSFC methods (i.e. > 70% for carbohydrates and organic acids, > 80% for amino acids, quaternary amines, sulphates/sulfonated metabolites and nucleosides). All the above-mentioned metabolites share a high polarity and were eluted in UHPSFC with a relatively high percentage of organic modifier in the mobile phase. It is also important to keep in mind that these polar metabolites were successfully analyzed, thanks to the presence of water and additives in the mobile phase, as already discussed in [13].

The use of unconventional SFC conditions (up to 100% organic modifier) is also not incompatible with the analysis of lipophilic metabolites. Indeed, high detectability percentages (>70%) were also found for lipophilic compounds such as steroids and lipids/lipid related metabolites, which were eluted at the beginning of the gradient with low organic modifier percentages. However, such detectability percentages were obtained after choosing a different solubilization solvent than what was chosen at the beginning of the experiments. A mixture of 95/5 MeOH/H_2O *v/v* was initially used as a solubilization solvent to obtain the stock solutions at 25 µg/mL for steroids and lipids/lipid related metabolites. These stock solutions, once diluted, were analyzed with the UHPSFC–HRMS analytical method and gave lower percentages of detectability (52% for steroids, 54% for lipids/lipid related metabolites). Such low values were unexpected, as these classes are well-known to be successfully analyzed using standard UHPSFC–HRMS conditions. Therefore, it was decided to use a different sample diluent, as the one previously used might have been not well adapted. The choice fell on dichloromethane, since it is able to dissolve lipophilic substances and its aprotic characteristics are suitable in providing good peak shapes under SFC conditions [26]. Its use was successful, as it enables to enhance the detectability percentages for steroids and lipids/lipid related metabolites. To further increase this percentage, another ionization technique (such as APCI or APPI) should be tested as some metabolites belonging to these categories are too lipophilic for ESI ionization mode.

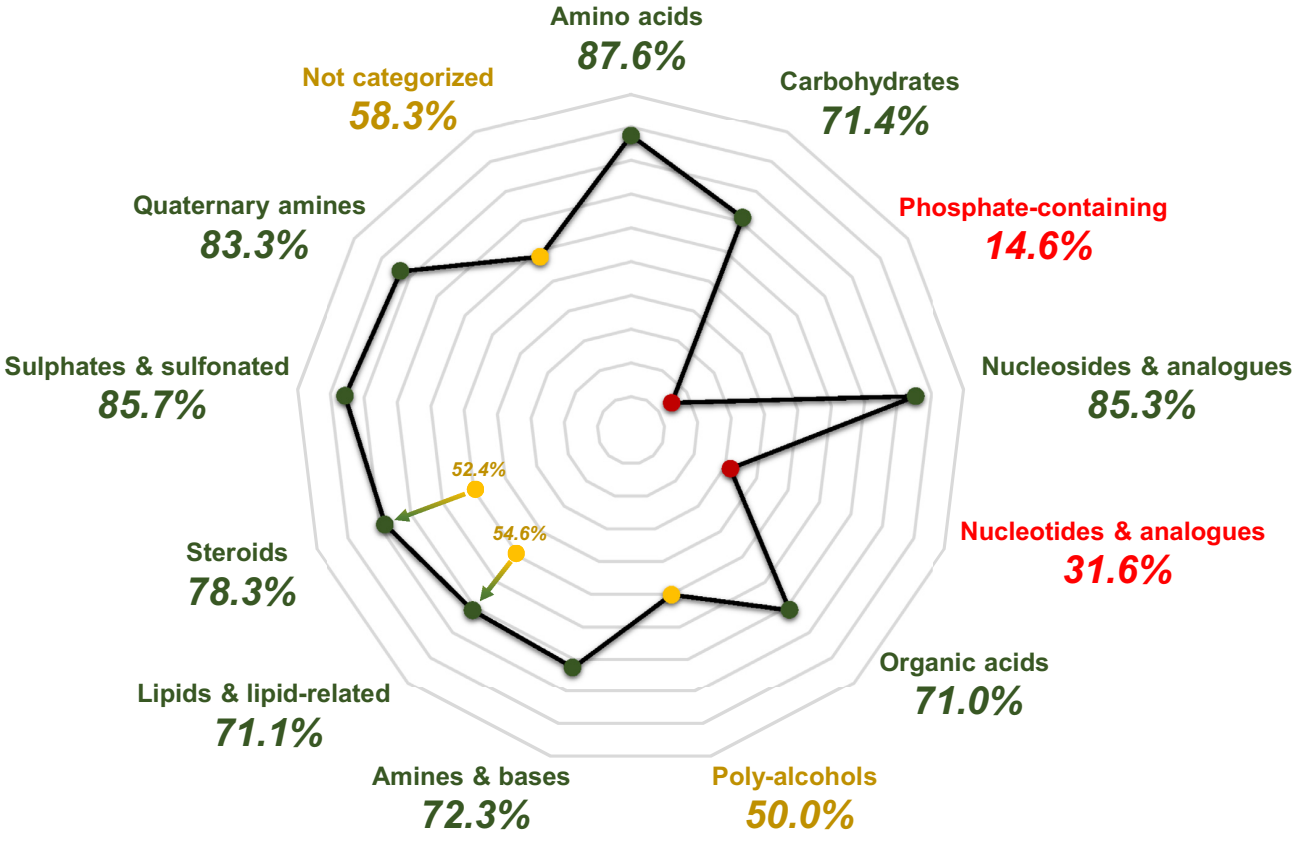

Fig. 7. Spider graph depicting the detectability percentages of each class of metabolites present in the Sigma MSMLS.

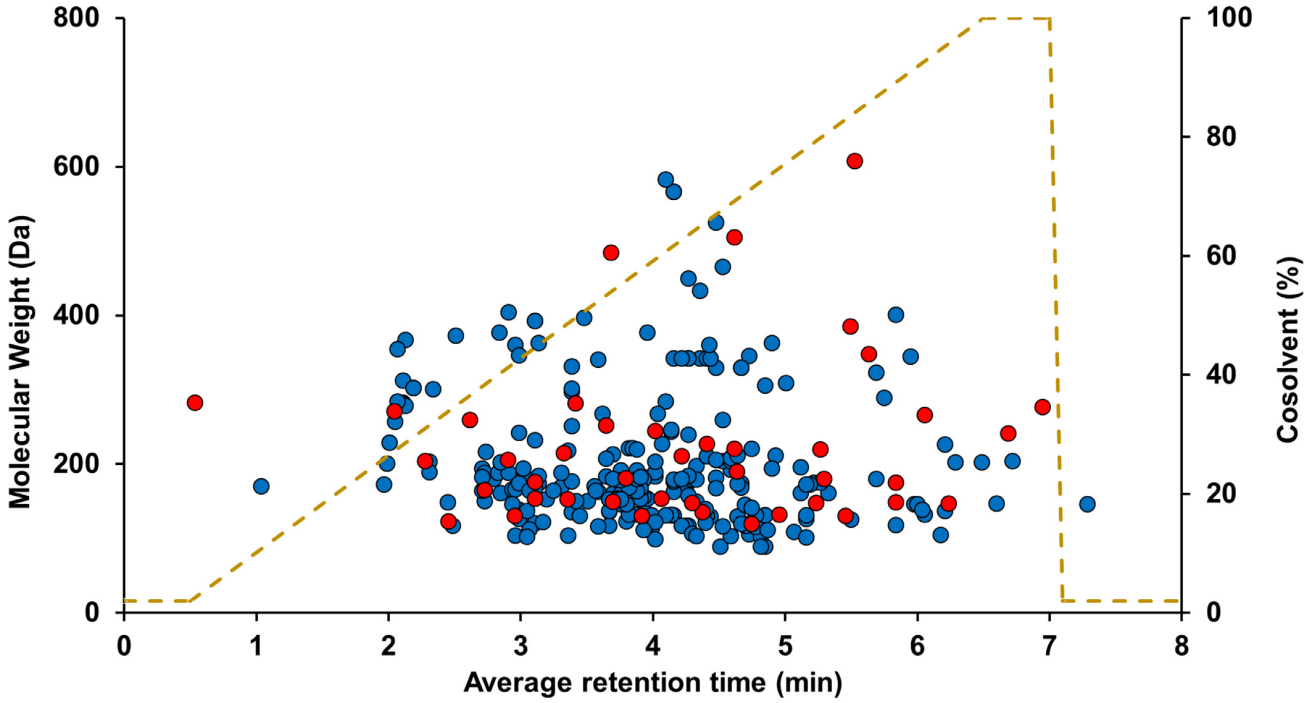

Fig. 8. Scatter plot of each detected metabolite from the Sigma MSMLS, according to their average retention times (min), molecular weights (Da) and percentage of cosolvent needed for their elution.

However, not all classes were easily detected under UHPSFC–HRMS conditions. Despite the several efforts being made to improve the detectability of hydrophilic compounds in UHPSFC, low success rates were observed for two specific categories of metabolites namely nucleotides and analogues (32%) and phosphate containing compounds (15%). The presence of one or more phosphate groups seems to be highly detrimental under UHPSFC–HRMS conditions. This becomes even more obvious when comparing the behavior of two families of compounds which differ only in the presence of phosphate groups, namely nucleosides (no phosphate) and nucleotides (one or more phosphate). There are several possible hypotheses to explain these negative results: a possible precipitation of the metabolites might happen due to the incompatibility of such substances with the UHPSFC mobile phase, especially at the beginning of the gradient profile where a high proportion of supercritical CO_2 is present. In addition, the possible adsorption phenomenon of phosphorylated compounds on the walls and frits of the stainless-steel column could occur, due to the chelation phenomenon generated by the phosphate groups to the metallic surface. Lastly, it is also possible that the phosphate metabolites are simply too much retained and cannot be eluted from the UHPSFC column under the selected conditions. As demonstrated by others [27], the use of less orthodox gradient profiles enabled the successful analysis of nucleosides and, more important, of nucleotides as well.

Despite the negative results obtained for some categories of metabolites, the overall performance of UHPSFC–HRMS with this metabolomic library can be considered as excellent. Besides the possibility to successfully analyze a wide range of metabolites, it is also important to notice that all the detected metabolites presented a relatively high retention factor. This is illustrated in Fig. 8, where the average retention times of each metabolite successfully detected from the Sigma Metabolite Library (blue points) and those belonging to the original set of 49 metabolites previously used (red points) was plotted over the gradient profile used in this study. As shown, the early eluted lipophilic compounds are all sufficiently retained (only one metabolite, oleic acid, eluted during the initial isocratic hold close to the column dead time of 0.5 min), while the most hydrophilic compounds are all eluted during the gradient (only one metabolite, deoxycarnitine, eluted after the gradient). This observation is certainly one of the most important point to consider, when evaluating the implementation of UHPSFC–HRMS in the field of metabolomics. Indeed, unlike the other well-established chromatographic techniques such as RPLC and HILIC, which suffer from poor retention of hydrophilic (for RPLC) or lipophilic (for HILIC) metabolites, respectively, UHPSFC is able to successfully analyze all these compounds within the same run. Such interesting retention profile is due to the unique interaction mechanism in UHPSFC, consisting mostly of H-bond interactions between the analytes and the stationary phase. Since almost all metabolites can generate such interactions, UHPSFC can be considered as a highly generic analytical strategy, allowing to ensure a good retention profile from an extremely diverse pool of metabolites, from lipids to sugars and nucleosides, with identical analytical conditions.

4. Conclusion

In this study, the potential use of UHPSFC, coupled to a HRMS, for metabolomic analyses was assessed. Following a previous paper [11], the impact of biological matrices commonly analyzed in metabolomics, such as urine or plasma, was evaluated. The ME generated by those biological samples resulted in a limited number of compounds suffering from ME in both matrices (30% in plasma; 25% in urine). Ion suppression was the main source of ME for urine, while in plasma the presence of a more complex profile of endogenous compounds translates into the presence of both ion suppression (10% of metabolites) and, in a major form, ion enhancement (20%). The quality of MS/MS spectra was then considered. It was observed that 63% of metabolites do not suffer from the presence of matrix-related interfering compounds; while 21% seem to be influenced only in one type of biological matrix, and the third category (16% of the total metabolites) presents interferences whatever the matrix. The retention time repeatability of metabolites in these two biological matrices was also evaluated over a period of three weeks. The extremely low values of average RSDs calculated in all conditions (0.3 - 0.5%) represent another demonstration of how modern UHPSFC has evolved into a stable and robust technique, with performance very similar to the well-established UHPLC. Finally, the developed strategy was applied to a large library of metabolites. Almost 600 metabolites were analyzed, with a detection success rate of 66%. This study highlights how the developed UHPSFC–HRMS method has now proven to be quite powerful in detecting heterogenous families of metabolites using identical analytical conditions, from highly polar compounds to very lipophilic substances. Moreover, the peculiar UHPSFC retention mechanism allowed to obtain a very good retention profile for all detected metabolites, with enough retention for the most hydrophobic compounds and enough elution strength to successfully elute, the most hydrophilic metabolites. All these results confirm that UHPSFC–HRMS might be potentially considered as a valid alternative to the already established chromatographic techniques for metabolomic studies. As future perspectives, it is now imperative to further develop applications based on the analysis of real-life samples, to build specific database integrating UHPSFC retention factors as well to push forwards some applications and implementation in the field of targeted metabolomics.

Declaration of Competing Interest

None.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2020.461021.

CRediT authorship contribution statement

Gioacchino Luca Losacco: Writing - original draft, Methodology, Investigation. **Omar Ismail:** Writing - review & editing, Methodology, Investigation. **Julian Pezzatti:** Writing - review & editing. **Víctor González-Ruiz:** Writing - review & editing. **Julien Boccard:** Writing - review & editing. **Serge Rudaz:** Supervision. **Jean-Luc Veuthey:** Supervision, Resources. **Davy Guillarme:** Supervision, Project administration.

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II.5 UHPSFC for peptide analysis

II.5.1 Advantageous use of MSA in UHPSFC using a water-rich modifier for peptide analysis

Peptides are a class of therapeutic agents which have generated a relatively high interest from different pharmaceutical companies, due to their presence and key role in several biological processes in living organisms. Therefore, in the past 15-20 years there has been an increased availability, on the market, of both endogenous peptides as well as synthetic ones. LC has been the gold standard in the context of peptide analysis, but the need to develop orthogonal methods which can guarantee the same, if not even better performance, as well as faster analysis is always present.

In this article, the performance of UHPSFC for the analysis of synthetic and commercially available peptides have been assessed. Initially, the chromatographic method was developed and optimized; afterwards, a systematic comparison against RPLC has been made, using UV and MS detectors. Finally, a successful method transfer from analytical to semi-preparative scale was performed in SFC.

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Expanding the range of sub/supercritical fluid chromatography: Advantageous use of methanesulfonic acid in water-rich modifiers for peptide analysis

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ABSTRACT

The aim of this work was to expand the applicability range of UHPSFC to series of synthetic and commercialized peptides. Initially, a screening of different column chemistries available for UHPSFC analysis was performed, in combination with additives of either basic or acidic nature. The combination of an acidic additive (13 mM TFA) with a basic stationary phase (Torus DEA and 2-PIC) was found to be the best for a series of six synthetic peptides possessing either acidic, neutral or basic isoelectric points. Secondly, methanesulfonic acid (MSA) was evaluated as a potential replacement for TFA. Due to its stronger acidity, MSA gave better performance than TFA at the same concentration level. Furthermore, the use of reduced percentages of MSA, such as 8 mM, yielded similar results to those observed with 15 mM of MSA. The optimized UHPSFC method was, then, used to compare the performance of UHPSFC against RP-UHPLC for peptides with different pI and with increasing peptide chain length. UHPSFC was found to give a slightly better separation of the peptides according to their pI values, in few cases orthogonal to that observed in UHPLC. On the other hand, UHPSFC produced a much better separation of peptides with an increased amino acidic chain compared to UHPLC. Subsequently, UHPSFC-MS was systematically compared to UHPLC-MS using a set of linear and cyclic peptides commercially available. The optimized UHPSFC method was able to generate at least similar, and in some cases even better performance to UHPLC with the advantage of providing complementary information to that given by UHPLC analysis. Finally, the analytical UHPSFC method was transferred to a semipreparative scale using a proprietary cyclic peptide, demonstrating excellent purity and high yield in less than 15 min.

1. Introduction

Peptides and peptide-like drugs are compounds which typically generated a lot of interest within the pharmaceutical industry. Their presence in several key biological processes makes them an interesting class of molecules from which new drugs could be developed [1,2]. There have been several developments in the use of peptides as therapeutic agents: originally, they were simply used in replacement therapies, when patients lacked a specific peptide in their organism [3,4]. A classic example of this strategy is the administration of insulin to patients suffering from type 1 diabetes [3]. Subsequently, synthetic analogs of different peptides already present in the human body came along [5,6]. However, peptides present several issues as drugs, mainly related to their pharmacokinetic properties [7,8], because of their low bioavailability due to their size, up to 5000 – 6000 Da for peptides with an amino acidic sequence of 40–50 amino acids, as well as an facile metabolism [9]. To improve their properties, modern synthetic peptides have started to differ, from a structural point of view, from their biological precursors, including new functional groups in their structure (i.e. polymers and fatty acids) introduced to develop a better bioavailability via their oral formulation [10,11].

The analytical strategy to characterize this class of molecules has revolved on the use of ultra-high performance liquid

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chromatography (UHPLC) as the preferred technique, mainly in reversed-phase mode (RPLC) [12–14]. Its ease of use, high throughput capacity and ability to couple with ultraviolet (UV) detector and, more importantly, mass spectrometry (MS) made it a popular technique for peptide analysis [15–17]. Despite the advantages of UHPLC-UV-MS, a demand for greener, faster and complementary analytical techniques is always present [18]. Among them, one of the most promising strategies is ultra-high performance supercritical fluid chromatography (UHPSFC). Thanks to the development of dedicated sub-2 µm stationary phases, as well as the release of chromatographic systems able to withstand the backpressures generated by these columns, UHPSFC has shown a great potential as a complementary alternative to UHPLC. This was possible thanks to the use of a mobile phase consisting in a mixture of supercritical carbon dioxide with polar organic modifier [19]. Moreover, it presents an easy hyphenation to various detectors such as UV and MS [20] and can provide fast analyses as the mobile phase presents low viscosity, enabling higher flow-rates without experiencing high backpressures. Finally, a high degree of orthogonality exists between UHPSFC and UHPLC, especially with the RPLC mode [21].

The analysis of peptides in UHPSFC is described in the literature, and there have already been studies demonstrating the use of UHPSFC for their analysis [22–25]. However, a systematic comparison between UHPSFC and UHPLC has not been made until now. This is probably because UHPSFC is difficult to use for the analysis of highly polar compounds having high molecular weight (partial elution from the column, solubility issues, distorted peaks…). Nonetheless, in the last 2–3 years a new trend appears in UHPSFC, consisting in the use of gradient profiles reaching percentages of organic modifier up to 90–100% [26–28]. Furthermore, the addition of water, up to 5–7% in the organic co-solvent has enabled UHPSFC to give improved performance in the analysis of polar and ionized metabolites, as it increases the elution strength of the mobile phase [28,29]. These new trends in UHPSFC could, therefore, reinvigorate its applicability for the analysis of peptides.

The aim of this study was to evaluate the performance of UHPSFC, coupled to different detectors (UV and MS), for the analysis of a series of synthetic and therapeutic peptides. Different chromatographic aspects, such as retention, selectivity and peak shape, as well as compatibility with MS detection and, finally, scale-up to the preparative scale, have been investigated. The impact of peptide isoelectric point, hydrophobicity and amino acids chain length, on the UHPSFC separation was assessed. A systematic comparison to UHPLC in the RPLC mode was also performed with the goal of highlighting possible advantages and disadvantages of the newly developed method.

2. Materials and methods

2.1. Chemicals, reagents and sample preparation procedures

For all experiments performed at the University of Geneva, methanol (MeOH) and acetonitrile (ACN) of OPTIMA LC-MS grade and water (H_2O) of UHPLC grade were purchased from Fischer Scientific (Loughborough, UK). Carbon dioxide (CO_2) of 4.5 grade (99.995% purity level) was purchased from PanGas (Dagmerstellen, Switzerland). Metanil yellow and methyl orange, lysine, arginine, aspartic acid, glutamic acid, ammonia solution at 25% of MS grade, trifluoroacetic acid (TFA) of MS grade and methanesulfonic acid (MSA) at a purity level of 99.5% or higher were purchased from Sigma-Aldrich (Buchs, Switzerland). Synthetic peptides 1N, 2N, 1B, 2B, 1A, 2A, 6mer, 9mer, 12mer, 15mer, 18mer and 21mer at a purity level of $\geq$ 95% have been purchased from GenScript Biotech (Leiden, Netherlands). More information regarding their amino acid sequences, molecular weights as well as predicted isoelectric points (pI) and GRAVY numbers are provided in Table 1. GRAVY number is a measure of the grade of hydrophilicity of a protein/peptide based on its hydropathy index, a value which varies between −2 to 2 for most proteins; the higher the hydropathy index, the higher the hydrophobicity. GRAVY number and pI values were obtained using the ProtParam tool available on the proteomic server ExPASy [30,31]. Commercial pharmaceutical formulations of liraglutide, leuprorelin, glucagon, cyclosporin A, eptifibatide and linaclotide (Table 1) have been purchased from the hospital pharmacy at the Geneva University Hospitals (HUG, Geneva, Switzerland).

For all purification experiments, methanol (HPLC Grade) and water (HPLC grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Methanesulfonic acid (MSA), 99% extra pure was purchased from ARCŌS Organics (Morris Plains, NJ, USA). The cyclic peptide was obtained in-house (Merck & Co., Inc., Kenilworth, NJ, USA). Bone dry-grade CO2 was obtained from Air Gas (New Hampshire, USA).

Details regarding the sample preparation and stress procedures used in this study can be found in the supplementary material.

Table 1
List of synthetic and commercial peptides used in this study.

Name	Sequence	MW (Da)	Number of amino acids	pI (predicted)	GRAVY number
Peptide 1N	Trp-Asn-Ser-Val-Lys-Tyr-Asp-Ile-Ser-Tyr-His-Thr	1512	12	6.74	−0.93
Peptide 2N	Ala-Tyr-His-Asp-Gln-Trp-Lys-Tyr-His-Phe-Cys	1497	11	6.95	−1.24
Peptide 1B	Trp-Gln-Ser-Thr-Tyr-His-Asp-Lys-Phe-Ala-Trp-Arg-Tyr	1788	13	8.50	−1.53
Peptide 2B	Phe-Lys-Asn-Ser-Tyr-His-Gln-Ile-Arg-Trp-Val-Tyr-Asn-Phe	1902	14	9.70	−0.86
Peptide 1A	Phe-Asn-Glu-Cys-Tyr-Arg-Ser-Asp-Ala-Tyr-Ser-Asn-Thr-Phe	1717	14	4.37	−0.96
Peptide 2A	Tyr-Asn-Ser-Phe-Asp-Glu-Trp-Lys-Cys-Thr-Phe-Ser-Trp	1713	13	4.37	−0.90
Peptide 6mer	Leu-Trp-His-Gly-Ser-Asn	713	6	6.74	−0.83
Peptide 9mer	Leu-Trp-His-Gly-Ser-Asn-Lys-Trp-Asp	1142	9	6.74	−1.48
Peptide 12mer	Leu-Trp-His-Gly-Ser-Asn-Lys-Trp-Asp-Asn-Gly-Gln	1441	12	6.74	−1.73
Peptide 15mer	Leu-Trp-His-Gly-Ser-Asn-Lys-Trp-Asp-Asn-Gly-Gln-Trp-Ser-Asn	1829	15	6.74	−1.73
Peptide 18mer	Leu-Trp-His-Gly-Ser-Asn-Lys-Trp-Asp-Asn-Gly-Gln-Trp-Ser-Asn-Gly-Thr-Gln	2115	18	6.74	−1.69
Peptide 21mer	Leu-Trp-His-Gly-Ser-Asn-Lys-Trp-Asp-Asn-Gly-Gln-Trp-Ser-Asn-Gly-Thr-Gln-Ala-Asn-Ser	2387	21	6.74	−1.57
Liraglutide	His-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys(γ-Glu-palmitoyl)-Glu-Phe-Ile-Ala-Trp-Leu-Val-Arg-Gly-Arg-Gly	3751	32	4.96	−0.36
Leuprorelin	pGlu-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-NHEt	1209	9	8.75	−0.52
Glucagon	His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Ser-Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Met-Asn-Thr	3483	29	6.75	−0.99
Cyclosporin A	Abu-Sar-Leu-Val-Leu-Ala-dAla-Leu-Leu-Val-Bmt	1203	11	NA	NA
Eptifibatide	Cys-hArg-Gly-Asp-Trp-Pro-Cys	832	7	3.80	−0.20
Linaclotide	Cys-Cys-Glu-Tyr-Cys-Cys-Asn-Pro-Ala-Cys-Thr-Gly-Cys-Tyr	1527	14	4.00	0.32

2.2. Chromatographic and MS instrumentations and conditions

At University of Geneva, for UHPSFC analyses, five different columns have been initially employed, namely Torus 2-PIC, Torus DEA, Torus DIOL, BEH silica (Waters, Milford, MA, USA), all packed with 1.7 µm fully porous silica particles, and Nucleoshell HILIC (Macherey-Nagel, Düren, Germany), packed with 2.7 µm superficially porous silica particles. All columns possess the same geometry of 100 × 3.0 mm I.D. A generic gradient was developed for the analysis of synthetic peptides, from 10 to 100% organic modifier in the mobile phase over 7 min, followed by an isocratic hold at 100% of co-solvent for 1 min, then a return to initial conditions in 0.1 min, and a final isocratic step with 10% of organic modifier for 2.9 min, giving a total run time of 11 min (section 3.1.). Organic modifier employed at this stage was a mixture of MeOH/H_2O 95:5 *v/v* containing either 13 mM (0.1%) TFA, 15 mM (0.1%) MSA or 52 mM (0.2%) NH_4OH. Flow-rate was fixed at 0.7 mL.min^{-1}. Following this preliminary step, an optimized method for the analysis of synthetic peptides was developed and used in the second part of the study (section 3.2), based on the Torus 2-PIC stationary phase with mixture of MeOH/H_2O 95:5 *v/v* + 8 mM MSA as the co-solvent. The optimized method follows a different gradient profile, starting from 30 to 80% organic modifier over 5 min, then an isocratic step at 80% of co-solvent for 0.5 min, followed by a return to initial conditions in 0.1 min and a second isocratic step of 1.9 min for a total analysis time of 7.5 min. Flow-rate was fixed, in this case at 0.9 mL.min^{-1}. For the commercially available peptides (i.e. liraglutide, leuprorelin, glucagon, linaclotide and eptifibatide), a modified version of the optimized gradient was employed: start at 35% co-solvent, reaching 90% in 5 min, then an isocratic step at 90% of co-solvent for 0.5 min, followed by a return to initial conditions in 0.1 min and a second isocratic step under these conditions of 1.9 min for a total analysis time of 7.5 min. For cyclosporin A, a third gradient was chosen, starting from 2 to 40% over 5 min, with an isocratic step at 40% of co-solvent for 0.5 min, then return to initial conditions in 0.1 min and a second isocratic step for 1.9 min, giving a total run time of 7.5 min.

Under UHPLC conditions, a 50 × 2.1 mm I.D. BEH C_{18} stationary phase packed with 1.7 µm fully porous particles (Waters) was used. Mobile phase A was H_2O + 13 mM TFA, while mobile phase B was ACN + 13 mM TFA. An optimized gradient was employed for all synthetic and therapeutic peptides (with the only exception of cyclosporin A), consisting in a 5 min gradient from 5 to 65%B, a hold up for two minutes at 65% B, then a return to initial conditions in 0.1 min and an isocratic hold for 1.9 min at 5% for a total run time of 9 min. For cyclosporin A the gradient time and total run time were the same, however the highest percentage of B reached during the gradient was 95%. In all these conditions, the flow-rate was fixed at 0.4 mL.min^{-1}.

The column screening consisted of eight different stationary phases, namely Chiralpak IC and Chiralcel OZ (both of geometry of 100 × 4.6 mm I.D. – 3.0 µm fully porous particles); Chiralcel OJ, Chiralpak IG and DCpak P4VP (all with geometry of 150 × 4.6 mm I.D. – fully porous particle sizes of 3.0 µm for Chiralcel OJ and of 5.0 µm Chiralpak IG and DCpak P4VP) from Chiral Technologies (West Chester, PA, USA); CELERIS 4EP from Regis Technologies (Morton Grove, IL, USA) and Torus DEA and Torus 2-PIC from Waters Corp. (Milford, MA, USA), all with the geometry of 250 × 4.6 mm I.D. and packed with 5.0 µm fully porous particles. SFC screenings were carried out on the diverse set of columns described in the above section by gradient elution at a flow rate of 2 mL.min^{-1} with the backpressure regulator (BPR) set at 103 bar (1500 psi). The SFC eluents consisted of CO_2 and organic modifier, which consisted of MeOH/H_2O 95/5 *v/v* + 8 mM MSA. The mobile phases were programmed as follows: 35% B at 0 min, linear gradient from 35% to 90% B in 5 min, a hold at 90% B for 0.5 min, then return to 35% B in 0.1 min and finally hold at 35% B for 1.9 min. The PDA scans from 190 to 400 nm and the chromatogram is extracted at 210 nm. The MS scans the mass range of 100 to 1200 with a sampling frequency of 2 Hz, cone voltages of 10 and 50 V in ESI (+) and a cone voltage of 10 V in ESI (-). Preparative SFC purification was performed on a Waters Torus 2-PIC 30.0 mm x 250 mm, 5 µm column with a mobile phase of 35% MeOH/H_2O 95/5 *v/v* + 8 mM MSA / CO_2. The flow rate was 140 mL.min^{-1}, mobile phase and column oven temperature at 35 °C, back pressure regulator set to 103 bar (1500 psi), UV detection at 210 nm. The sample was prepared at 20 mg/mL in methanol with a load of 1 mL.

SFC analysis of the cyclic peptide was carried out on a Waters Torus 2-PIC 4.6 mm I.D. x 250 mm 5 µm column at a flow rate of 2 mL.min^{-1} with the backpressure regulator (BPR) set at 100 bar; The SFC eluent solvent was 40% MeOH/H_2O 95/5 *v/v* + 8 mM MSA / CO_2. The PDA scans from 190 to 400 nm and the chromatogram was extracted at 210 nm.

All information regarding the chromatographic and MS instruments conditions, as well as on the software employed for data treatment can be found in the supplementary material.

3. Results and discussion

3.1. Development of the UHPSFC chromatographic method

3.1.1. Impact of the additive nature on the stationary phase performance

To ensure the elution of peptides using a CO_2-based mobile phase, various parameters have to be considered. Firstly, the addition of water in the co-solvent seems necessary to ensure acceptable peak shapes as well as elution within reasonable time [25,32–34]. Secondly, additives are needed to further reduce the tailing factor and peak widths [23,24,35]. To choose the most appropriate stationary phase, a screening of the several chemistries available was often needed. Overall, analytical conditions for peptide analysis under SFC can be summarized as follows: a mixture of methanol and water as the organic co-solvent, in combination with an additive (in most cases TFA). However, the application of such conditions is mostly limited to the analysis of peptides with relatively short amino acidic sequences (often 10–12 or less) [22–24,32]. Therefore, the goal of the present study was to find conditions suitable for a wider range of peptides, through the screening of different stationary phase chemistries, in combination with the use of acidic and basic additives. Such a screening strategy was firstly applied to a series of synthetic peptides described in Table 1 (peptides 1 N, 2 N, 1B, 2B, 1A and 2A). These peptides all possess a sequence of a length between 11 – 14 amino acids and with a molecular weight ranging from 1500 to 1900 Da. Furthermore, these different peptides possess either an acidic (pI < 7), neutral (pI ≈ 7), or basic nature (pI > 7) and they all possess an important polar character (GRAVY number between −1 and −2). Indeed, compounds with these properties have always been challenging to analyze under UHPSFC conditions, as they are strongly retained on the (polar) stationary phase, and poorly soluble in mobile phases with a predominant presence of supercritical CO_2. Each stationary phase (i.e. Torus 2-PIC, Torus DEA, Nucleoshell HILIC, Torus DIOL, BEH silica) was tested with the same organic modifier composition (MeOH/H_2O 95:5) in which either 13 mM (0.1%) of TFA or 52 mM (0.2%) of NH_4OH was added. In Fig. 1a, a table summarizing the data is presented. Stationary phases with a "basic" nature (having one or more positively charged functional groups) are those providing the best results, yielding complete elution of all synthetic peptides with good peak shape, as illustrated in Fig. 1b for peptides 1B and 2A on the Torus 2-PIC. Between the Torus 2-PIC and Torus DEA, no major differences were observed,

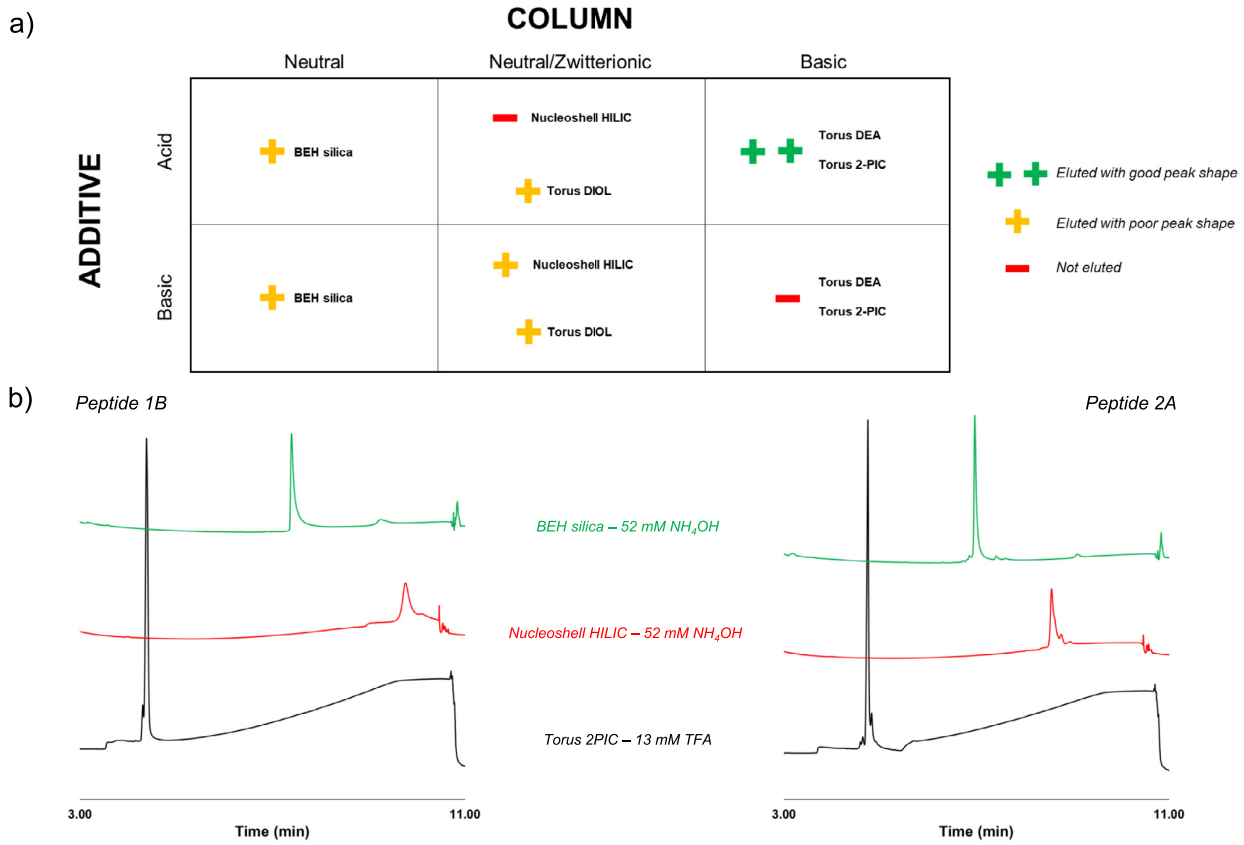

Fig. 1. a) A classification of the combination between the nature of the additive and the properties of the stationary phase chemistries evaluated in this study, on the quality of the chromatographic separation and elution for a series of synthetic peptides. b) Chromatograms, for peptides 1B and 2A, obtained on a "neutral", "zwitterionic" and "basic" stationary phase using the best combination between stationary phase and nature of the additive in the mobile phase.

but the Torus 2-PIC gave a slightly faster elution. As expected, these columns gave good results only when an acidic additive was used. The addition of 52 mM NH_4OH in the mobile phase provided a severe loss of performance on the two "basic" stationary phases (i.e. Torus 2-PIC and Torus DEA) (Fig. S1). The combination of a bare silica (BEH silica) stationary phase with acidic additive such as TFA, or even basic additives (52 mM NH_4OH) provided inferior performance to those witnessed on the Torus 2-PIC/DEA columns (Fig. 1a-b). With acidic peptides (peptide 2A), the BEH silica gave comparable peak shapes to that observed on the Torus 2-PIC (Fig. 1b), but did not for peptides with higher isoelectric points (peptide 1B – Fig. 1b). Finally, the two remaining columns employed in this study, namely the Torus DIOL (neutral) and Nucleoshell HILIC (zwitterionic), were those offering the worst performance overall. More specifically, the use of a zwitterionic stationary phase performed rather poorly with 13 mM TFA, while the addition 52 mM NH_4OH ensured the proper elution of peptides, but with extremely poor peak shapes as shown for peptides 1B and 2A (Fig. 1b). In conclusion, the combination of a column having basic properties (Torus 2-PIC) with an acidic additive (13 mM TFA) provided the best performance for all peptides and was kept for further evaluation.

3.1.2. Evaluation of MSA as a replacement of TFA

The use of TFA is quite widespread in the literature for peptide analysis, regardless of the considered chromatographic technique (UHPLC or UHPSFC). This additive, however, presents issues when coupling the chromatographic method to a MS detector, mostly due to its tendency to cause ion suppression in the ionization source. Moreover, its use does not always guarantee, in the case of UHPSFC, good chromatographic performance with peptides. The use of alternative additives that would either improve the MS compatibility or the chromatographic performance without sacrificing even further the MS sensibility is desirable. In this context, a recent article on the use of UHPSFC for the analysis of amino acids describes the use of a different additive, namely methanesulfonic acid (MSA), in substitution to TFA [28]. The use of MSA is not new in UHPSFC [36], and in this paper [28] the authors have highlighted a major improvement of the chromatographic performance in UHPSFC for the analysis of underivatized amino acids, in comparison with TFA. Moreover, authors have shown a compatibility of MSA-based mobile phases with MS detection. Therefore, it was decided to evaluate MSA instead of TFA for analyzing the same set of synthetic peptides previously used (i.e. 1N, 2N, 1B, 2B, 1A and 2A) on the Torus 2-PIC column. In Fig. 2, a comparison of 13 mM TFA vs. 15 mM MSA for peptides with acidic, neutral and basic pI is shown. It is immediately visible how 15 mM MSA largely improves the quality of the separation under UHPSFC conditions, improving both peak widths and peak shapes. Moreover, a higher number of impurities, which were not detected with 13 mM TFA, are now visible with 15 mM MSA. In order to make the mobile phase even more MS friendly, lower percentages of MSA (8 mM and 4 mM) have been assessed on the same set of peptides (Fig. 3). The reduced percentage of MSA did not negatively impact the performance of the chromatographic method overall, and 8 mM MSA gave similar results to those observed with 15 mM of MSA. A further reduction to 4 mM MSA was still sufficient to ensure the proper elution of the peptides, but peaks widths were slightly larger, and selectivity

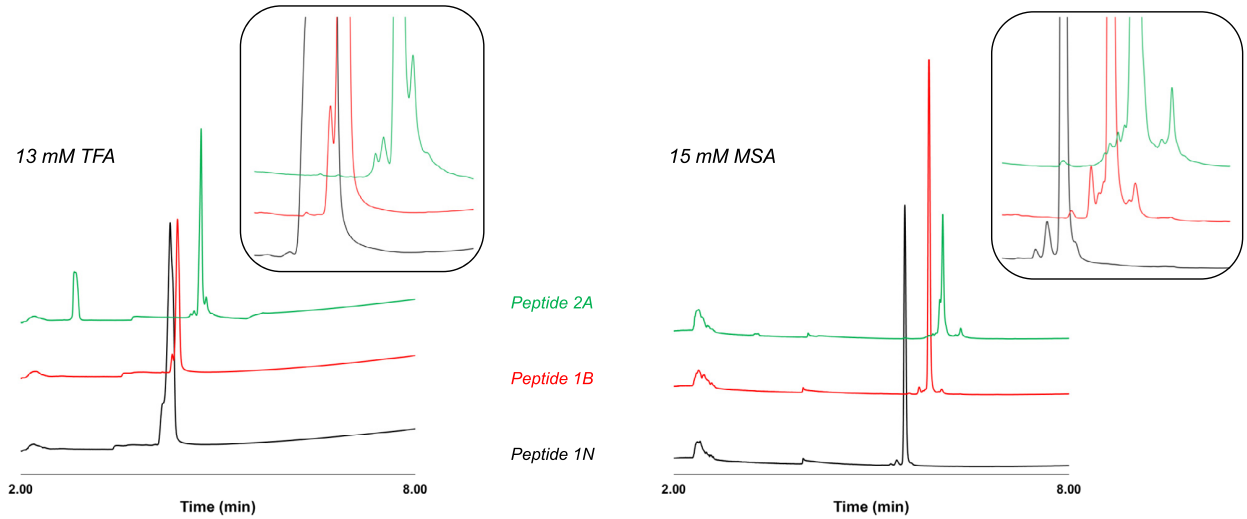

Fig. 2. Chromatograms, relative to peptides 1N, 1B and 2A, obtained on the Torus 2-PIC column with 13 mM TFA (left) or 15 mM MSA (right) as additives in the organic co-solvent.

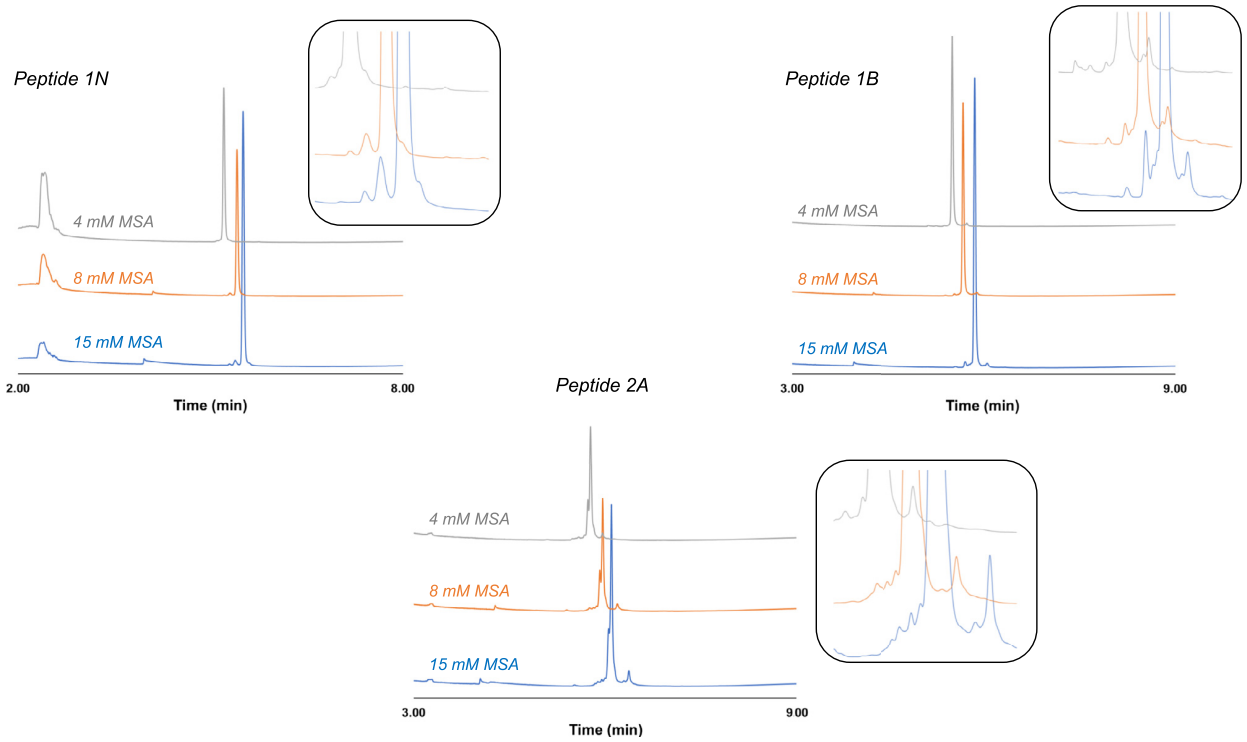

Fig. 3. A comparison of chromatograms obtained by using different percentages of MSA (4 mM – 8 mM – 15 mM) in the organic co-solvent on a series of three peptides (1N – 1B – 2A) on the Torus 2-PIC stationary phase.

was reduced compared to 15 mM and 8 mM MSA. Consequently, it was decided that 8 mM MSA was the best compromise for the UHPSFC method.

The chemical properties of this additive could explain the better chromatographic performance obtained for peptide analysis in comparison to TFA. Indeed, MSA is a strong organic acid with a very low pK_a value ($pK_a \approx -1.9$), in comparison with TFA (pKa $\approx$ 0.5). This important difference in the acidity scale might generate, some potential changes in the apparent mobile phase pH (pH_{app}). Due to the peculiar nature of the mobile phase generally employed in UHPSFC, consisting in a mixture of supercritical CO_2 with a polar organic modifier (generally methanol), a straightforward discussion of the mobile phase pH is almost impossible. However, in a recent article [37], the prediction of the pH_{app} in UHPSFC mobile phases was made thanks to the use of colorimetric pH indicators. In this work, the authors discovered that UHPSFC mobile phases possessed an average pH of 4–5, reaching lower values with the employment of acidic additives, such as TFA. Using the same strategy, an evaluation of the mobile phase acidity, with 8 mM MSA and 7 mM TFA, was carried out (Fig. 4), using 50% of supercritical CO_2 and 50% of co-solvent as the mobile phase. A reference solution without any additive in the co-

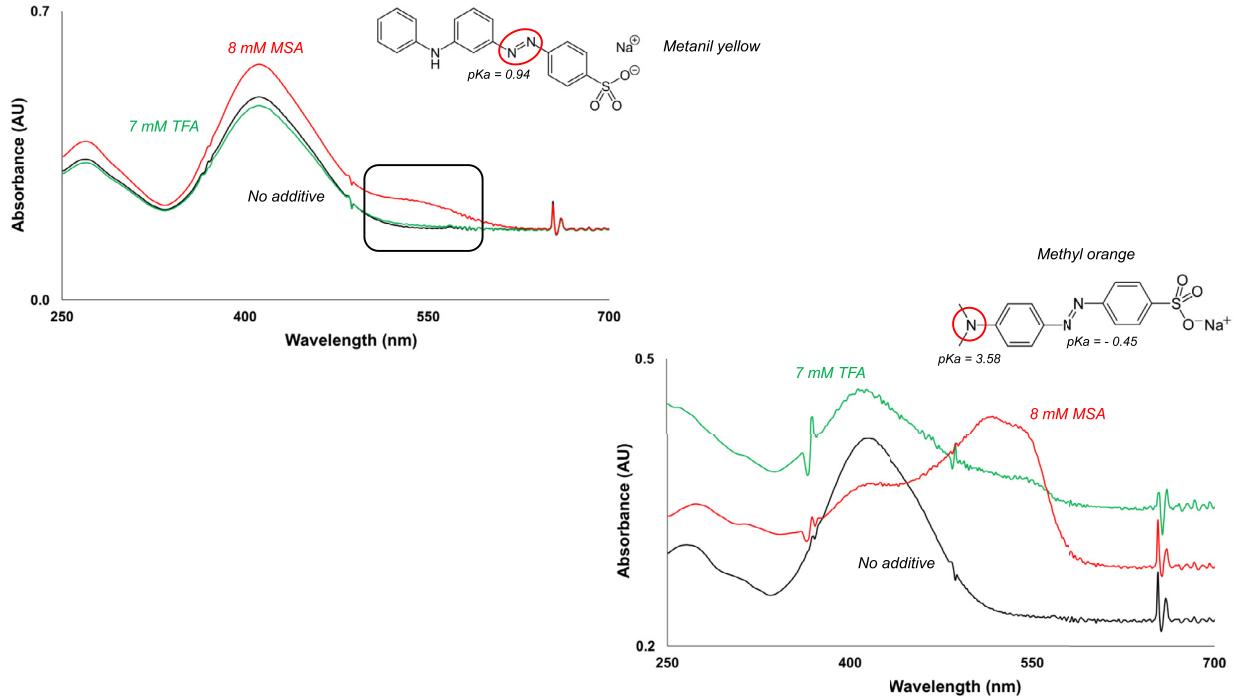

Fig. 4. UV spectra recorded for metanil yellow (left) and methyl orange (right) using 50/50 CO_2:B as the mobile phase, with B being: MeOH:H_2O 95/5 *v/v* (black trait), MeOH:H_2O 95/5 *v/v* + 8 mM MSA (red trait) and MeOH:H_2O 95/5 *v/v* + 7 mM TFA (green trait). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

solvent was also considered. The UV spectra recorded for two pH indicators, namely methyl orange ($pK_a \approx 3.6$) and metanil yellow ($pK_a \approx 0.9$) indicated that both additives were differently affected by the mobile phase acidity. This difference was already visible when methyl orange was used. Indeed, while no difference was observed, in the UV spectra, between the co-solvent without additive and with 7 mM TFA, a shift of the maximum absorbance towards higher wavelength was observed with 8 mM MSA (Fig. 4). This indicates a possible change in the protonation site present in the structure of the pH indicator. Surprisingly, a slight variation of the UV spectra was also observed for metanil yellow, a molecule with a much lower pK_a value (Fig. 4). It becomes therefore clear that MSA can generate a more acidic environment than TFA. The mobile phase pH_{app} seems to have a key role when considering the performance of UHPSFC for peptide analysis. The acidic conditions generated by 8 mM MSA can be sufficient to protonate all tested peptides, as their free carboxyl group at one end of the peptide chain (a weak acid) should be present in its protonated (neutral) form, while the free primary amine at the N-terminus should be increasingly present in its protonated form. The use of a "basic" column would also translate into a protonated stationary phase, under such pH conditions. Protonated molecules, such as the investigated peptides, would therefore experience an electrostatic repulsion with the stationary phase possessing the same net charge, which seems to drastically improve peak shape and peak width (Fig. 2).

An interesting phenomenon was highlighted in Fig. 3: peptides showed a faster elution at lower MSA concentration. This phenomenon did not seem to affect either peak shape or peak width, but solely the retention. This trend is not similar with TFA (Fig. S2). In this case, the reduction of TFA concentration from 13 mM to 7 mM generated an increase in retention. This behavior is due to the ion pairing behavior of TFA. With MSA, however, the situation needs to be further clarified. As above-mentioned, MSA is a strong acid, which generates an acidic environment able to protonate all peptides and the basic groups at the surface of the stationary phase employed in UHPSFC. The increase of MSA concentration would translate in a further increase of the mobile phase acidity, but it also means that a higher number of methanesulfonate anions ($H_3C\text{-}SO_3^-$) should be present, allowing ion-pairing behavior of the MSA anion with the positively charged compounds. The positive charge present on the peptide is, therefore, better shielded, thus reducing the electrostatic repulsion with the positively charged stationary phase, explaining the higher retention. To confirm this hypothesis, a test with 4 amino acids, two of them having a basic functional group (lysine and arginine) and two with acidic functional groups in their structure (glutamic and aspartic acid), was performed on the Torus 2-PIC using 8 mM and 15 mM of MSA and also using TFA. While peptides containing lysine and arginine have experienced a noteworthy reduction of their retention time with lower MSA concentration, the two acidic amino acids showed no significant retention time variation when switching from 15 mM MSA to 8 mM of MSA (Table S1). Higher percentages of TFA, on the other hand, always producing decreasing retention (Table S1).

3.2. Comparison of UHPSFC-UV vs. UHPLC-UV for peptides analysis

3.2.1. Influence of peptide isoelectric point on selectivity

Following the first part of the study, an investigation of how UHPSFC might provide practical advantages over UHPLC (under RPLC conditions) for the analysis of peptides was performed. For this purpose, the six previously described synthetic peptides (i.e. 1N, 2N, 1B, 2B, 1A and 2A) possessing either acidic, neutral or basic isoelectric points were evaluated under UHPSFC and UHPLC conditions. Fig. 5 shows the corresponding chromatograms obtained with the two chromatographic techniques. Some trends become immediately visible. Firstly, the elution order is not the same: in

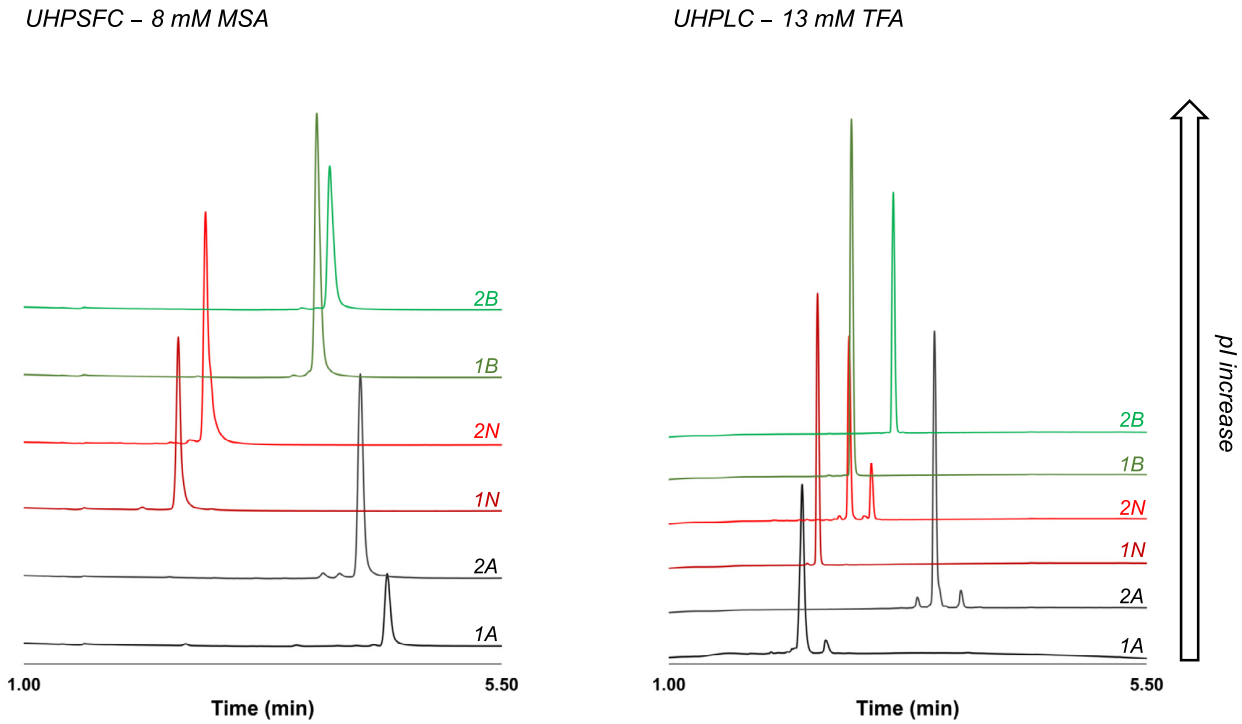

Fig. 5. Chromatograms obtained under UHPSFC-UV (left) and in UHPLC-UV (right) for the set of 6 synthetic peptides with increasing isoelectric point values (from bottom to the top: peptide 1A, 2A, 1N, 2N, 1B, 2B).

UHPLC acidic peptides show divergent retention, as seen with peptide 1A and 2A, being respectively the first and last eluted peptides among those tested. Neutral peptides, in UHPLC, are followed by basic peptides, but the separation can become hard to achieve (peptides 2N and 1B). While in UHPLC it was not always possible to obtain separate elution windows between peptides according to their pI value, as seen in the case of peptides 1A and 2A, with UHPSFC this was obtained (Fig. 5). Indeed, in UHPSFC peptides retention appears grouped according to pI: acidic, neutral and basic peptides possess their own elution windows, allowing a clear separation between these three groups for this example. The elution order is also different to the UHPLC one: neutral peptides are the least retained ones by the stationary phase, then the basic ones are eluted before those with an acidic pI. In reversed-phase UHPLC conditions, peptides are generally retained as hydrophobicity becomes higher, especially when TFA is employed in the mobile phase. In UHPSFC, the acidic environment protonates basic peptides to a higher degree compared to acidic peptides, but the presence of a positively charged stationary phase causes a stronger electrostatic repulsion phenomenon (as described in the previous section) with the basic peptides, thus reducing their retention. To clarify, however, why neutral peptides (1N and 2N) were even less retained under UHPSFC conditions compared to acidic and, more importantly, basic peptides, the influence of the chain length needs to be considered. A more detailed elucidation is given in the next section (3.2.2).

In summary, while the retention generally appears to follow the increase of pI in UHPLC, the retention behavior is different in UHPSFC. In the present example, the separation between peptides having different pI in UHPLC was challenging in some cases, as shown with peptides 2N and 1B On the other hand, UHPSFC was able to provide a satisfactory resolution (Fig. 5). While these results were all confirmed with the peptides at disposal, additional work needs to be performed with different samples.

3.2.2. Influence of peptide chain length on selectivity

Next to the impact of isoelectric point on retention and selectivity under UHPSFC and UHPLC, we also investigated the length of their amino acidic sequence. For this purpose, a new series of six synthetic peptides was employed (Table 1): peptide 6mer, 9mer, 12mer, 15mer, 18mer and 21mer. These peptides all share the same isoelectric point, to rule out the influence of this parameter. These peptides were then injected under the same optimized UHPSFC and UHPLC conditions used in section 3.2.1. Under UHPLC conditions, the elution of peptides with an amino acidic chain length comprised between 9 and 21 amino acids does not follow any order, as shown in Fig. 6. In addition, the selectivity between these different peptides was quite limited under these conditions and most of the peaks eluted within a narrow retention time window. In UHPSFC, the separation is much better, and peptides retention increases linearly with the sequence length (Fig. 6), without sacrificing the chromatographic resolution. The explanation of this retention behavior is quite obvious. Together with the increase in peptide length, there is also an increase in the number of polar groups on the molecule (amide bonding in particular), thus generating a higher retention on the polar stationary phase. Moreover, the electrostatic repulsion phenomenon would become less important as the positive charge on the peptide could be more delocalized when the peptide surface increases. In UHPLC, on the other hand, the apolar C18 stationary phase was not able to discriminate between shorter and longer peptides, even when using TFA as an ion pairing agent. This suggests that the lipophilicity of the peptides does not increase significantly with the increase of the length of their amino acidic chain for the samples taken into consideration, thus reducing chromatographic selectivity.

In section 3.2.1, it was highlighted that neutral peptides presented lower retention under UHPSFC conditions compared to basic ones. According to the electrostatic repulsion hypothesis, the opposite elution order would have been expected as basic peptides

should have a higher positive charge density compared to neutral peptides. However, an important parameter was left out from the discussion: peptides 1 N and 2 N have an amino acid chain length with 3 amino acids less compared to peptides 1B and 2B As it was just described, shorter peptides are less retained under UHPSFC conditions. This phenomenon could, therefore, influences the unexpected elution order previously observed between neutral and basic peptides, in combination with the different pI values possessed by these samples.

3.3. Application to the analysis of commercially available peptides

3.3.1. Analysis of linear and cyclic peptides

Various commercial therapeutic peptides (both linear and cyclic ones) were analyzed using the developed UHPSFC and the reference UHPLC methods. Furthermore, a MS detector was hyphenated to evaluate its performance with the developed UHPSFC method in comparison with the UHPLC one. Three linear (i.e. liraglutide, leuprorelin and glucagon) and cyclic (i.e. linaclotide, eptifibatide and cyclosporin A) therapeutic peptides have been employed in this part (Table 1). In addition, three different stressing procedures (i.e. acidic, basic or oxidative) were performed. Four samples for each peptide (control sample + 3 stressed sample) were, therefore evaluated in UHPSFC and UHPLC conditions. Chromatograms of control and stressed samples for each peptide with the two chromatographic techniques are shown in Fig. S3 for UHPSFC and Fig. S4 for UHPLC. All linear and cyclic peptides were eluted under UHPSFC conditions, while under UHPLC conditions, cyclosporine A could not be eluted under the generic conditions, even after a modification of the gradient profile to reach up to 95% ACN in the mobile phase. This result is not surprising, since cyclosporin A is a highly lipophilic cyclic peptide. In UHPSFC, a lower percentage of co-solvent in the gradient allowed the successful analysis of this particular sample. This result confirms the flexibility of UHPSFC at analyzing samples with a wide range of polarities on a single stationary and mobile phase.

A closer look to specific samples is shown in Figs. 7–8. In Fig. 7, a comparison between UHPLC and UHPSFC for control and stressed samples of leuprorelin is given (sequence of 9 amino acids). Both techniques provided a comparable chromatographic profile for the control sample, as well as the one stressed under acidic conditions, with impurity 1 ($[M + H]^+ = m/z$ 1101 under UHPLC-MS conditions, $[M + 2H]^{2+} = m/z$ 551 for UHPSFC-MS) always eluting prior to the main peak. The situation slightly varies with the basic conditions (Fig. 7). In this case, UHPSFC offered a better selectivity between impurities 2 ($[M + H]^+ = m/z$ 777), 1 ($[M + H]^+ = m/z$ 1101 under UHPLC-MS conditions, $[M + 2H]^{2+} = m/z$ 551 for UHPSFC-MS) and 3 ($[M + H]^+ = m/z$ 1194 under UHPLC-MS conditions, $[M + 2H]^{2+} = m/z$ 598 for UHPSFC-MS. Interestingly, in UHPSFC conditions, the elution order of impurities 1, 2 and 3 as well as leuprorelin was proportional to the molecular weights of the impurities. However, the chromatographic profile obtained after an oxidative stress was better resolved with the UHPLC method (Fig. 7), where a larger number of impurities was observed. The two new impurities 4 ($[M + H]^+ = m/z$ 1228 for UHPLC-MS, $[M + 2H]^{2+} = m/z$ 615 for UHPSFC-MS) and 5 ($[M + H]^+ = m/z$ 1245 for UHPLC-MS, $[M + 2H]^{2+} = m/z$ 622 for UHPSFC-MS) were eluted in opposite order by both methods.

Similar results were found with a second linear peptide, glucagon (Fig. 8). This 29 amino acid peptide possesses one of the longest amino acidic sequence among all samples tested in this work, as well as a relatively low GRAVY number, indicating a high polarity. Nonetheless, this peptide was eluted under UHPSFC conditions with a satisfactory peak shape using high amount of co-solvent (around 85% MeOH). Again, control as well as acidic stressed samples gave comparable profiles with both chromatographic techniques (Fig. 8). Impurities obtained after the addition of 0.1 M NaOH and hydrogen peroxide followed the same trends

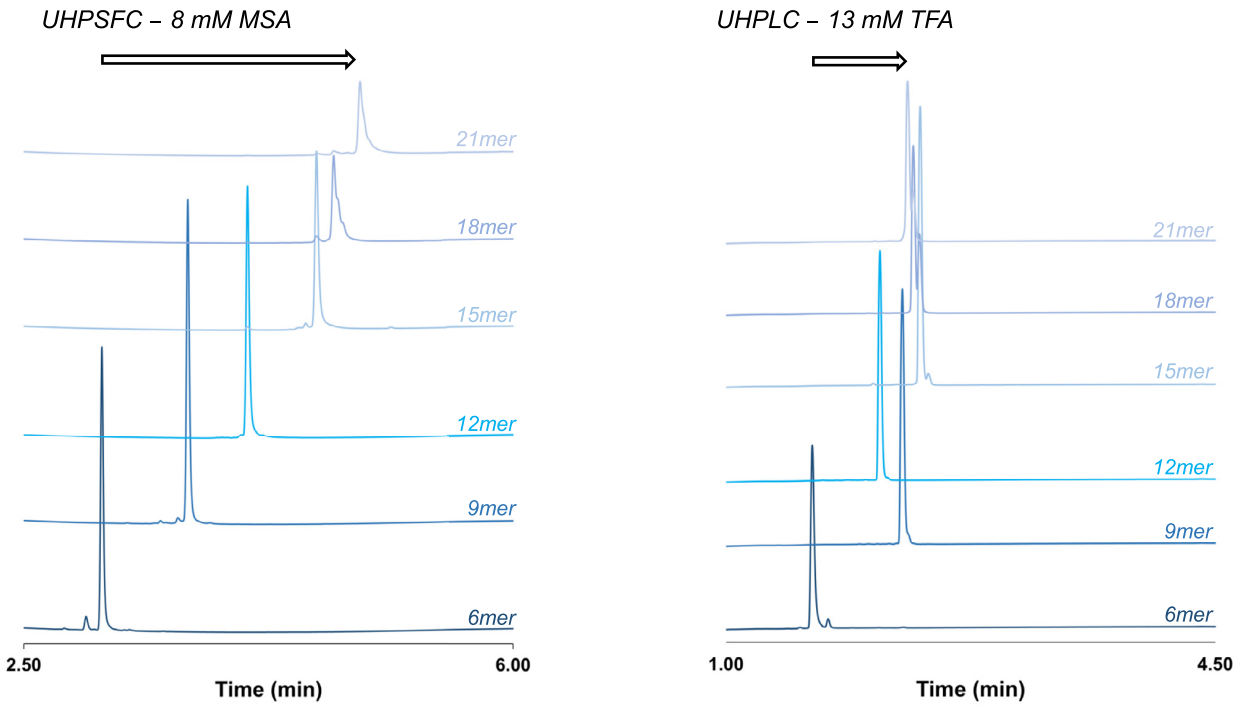

Fig. 6. Chromatograms obtained under UHPSFC-UV (left) and in UHPLC-UV (right), for the set of 6 synthetic peptides with increasing amino acidic chain length (from bottom to the top: peptide 6mer, 9mer, 12mer, 15mer, 18mer, 21mer).

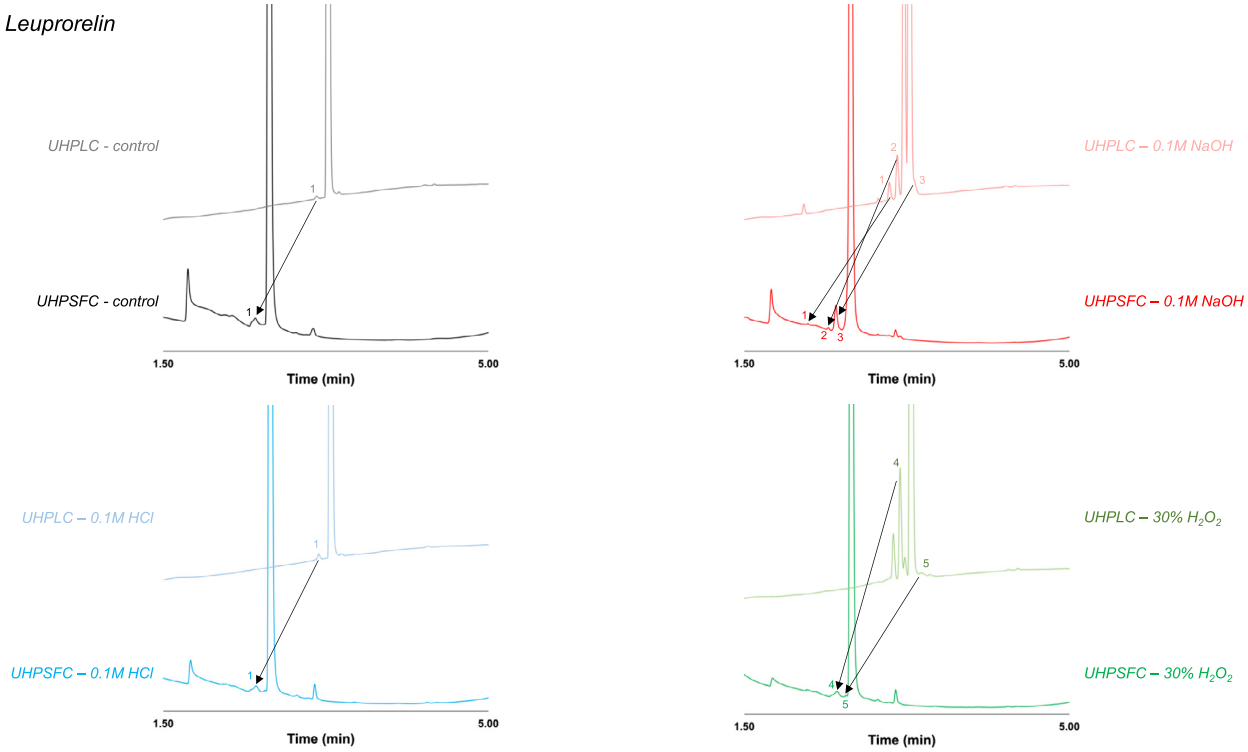

Fig. 7. Chromatograms obtained for leuprorelin and leuprorelin + impurities after exposure to different stress conditions in UHPSFC-UV-MS and UHPLC-UV-MS.

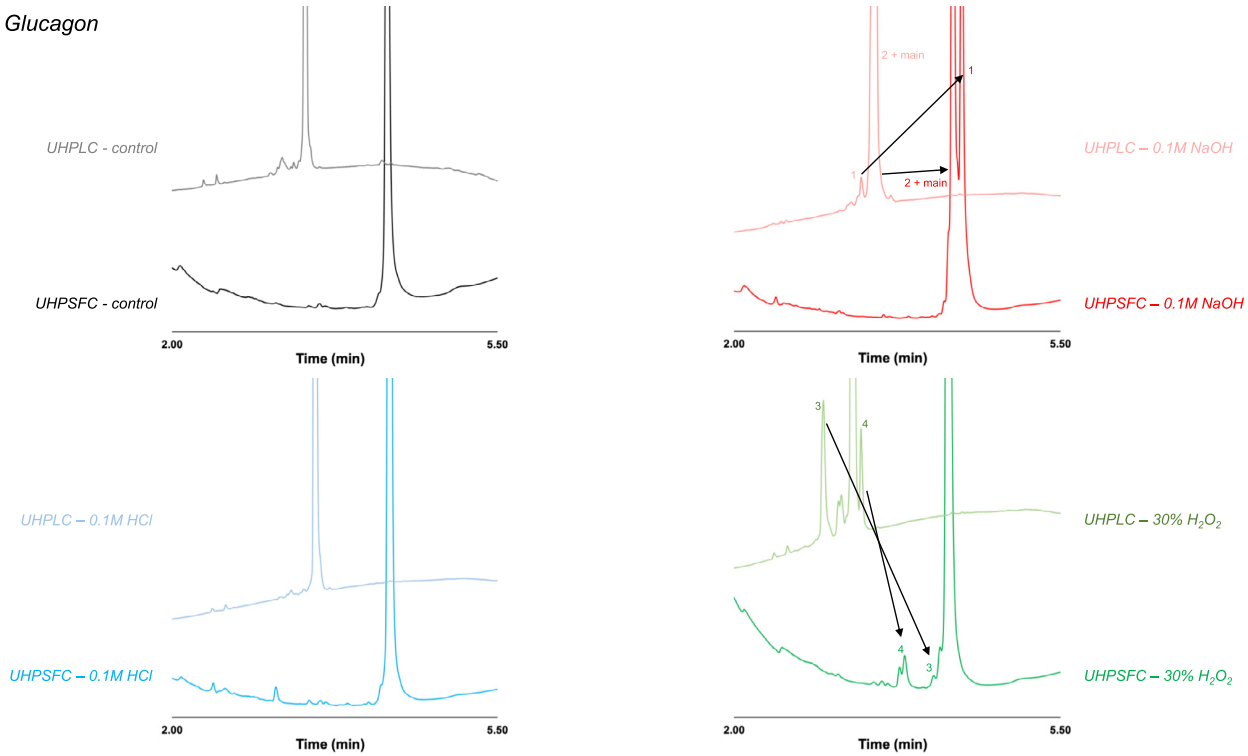

Fig. 8. Chromatograms obtained for glucagon and glucagon + impurities after exposure to different stress conditions in UHPSFC-UV-MS and UHPLC-UV-MS.

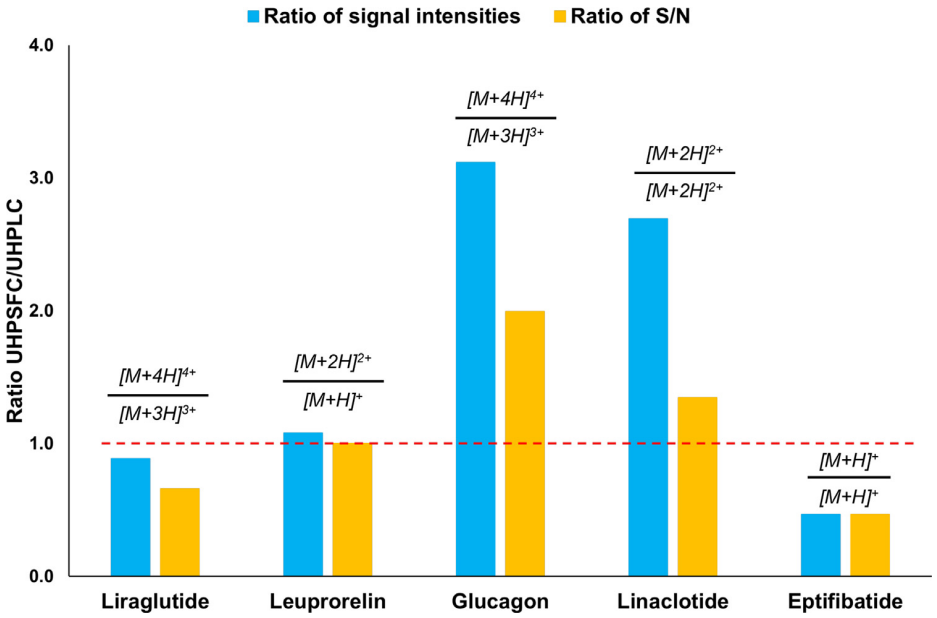

Fig. 9. Table representing the ratio between signal intensities (in blue) and signal-to-noise values (in yellow) obtained in UHPSFC-MS over UHPLC-MS conditions for five commercial peptides. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

as those previously reported for leuprorelin. Under basic conditions, glucagon impurity 1 ($[M + 3H]^{3+} = m/z$ 1319 for UHPLC-MS, $[M + 4H]^{4+} = m/z$ 990 for UHPSFC-MS) and 2 ($[M + 3H]^{3+} = m/z$ 1272 for UHPLC-MS, $[M + 4H]^{4+} = m/z$ 954 for UHPSFC-MS) eluted according to the length of their chain under UHPSFC-MS. The same behavior was also observed for impurities 3 ($[M + 3H]^{3+}$ - $[M + 4H]^{4+}$ of m/z 1179 and 885) and 4 ($[M + 3H]^{3+}$ - $[M + 4H]^{4+}$ of m/z 1168 and 881).

In Fig. S5 of the supplementary material, an example of a cyclic peptide composed of 7 amino acids, eptifibatide, is given. This peptide takes its characteristic cyclic structure after the formation of an intramolecular disulfide bridge between the two cysteine residues present in its chain. Once again, similar results have been observed when this compound was evaluated under UHPLC-MS and UHPSFC-MS conditions as to those previously discussed for linear peptides. While for the control sample, as well as under acidic stress procedure, no major differences were observed, while a larger number of impurities were observed after the exposure to 0.1 M NaOH. Impurities 1, 2 and 3 were better resolved from the main peak in UHPSFC conditions, and a higher number of impurities was visible compared to RP-UHPLC conditions. On the other hand, similarly to leuprorelin and glucagon, impurities produced after an oxidative stress were better resolved under UHPLC conditions.

Overall, this part demonstrated that UHPSFC was able, in almost all examples, to generate comparable performance to UHPLC, and gave complementary information (different elution behavior and selectivity).

3.3.2. Evaluation of MS sensitivity between UHPSFC vs. UHPLC

The use of MSA in the UHPSFC chromatographic method and its compatibility with MS detector was investigated. MSA is, indeed, a highly viscous organic acid with a relatively high boiling point (close to 170 °C, indicating potential issues in its application in chromatographic methods combined to mass spectrometers). Therefore, a systematic study was carried out, focusing on the ratio of the signal intensities, as well as of signal-to-noise values, obtained in UHPSFC and UHPLC for the commercial peptides previously employed (Fig. 9). Although MSA is not highly volatile, it is present at very low concentration in the UHPSFC method. Indeed, its average concentration in the gradient is equal to 4–5 mM (corresponding to 4 – 5 mM in the mobile phase), which is much lower than what is commonly employed in UHPLC (13 mM TFA). Consequently, as shown in Fig. 9, UHPSFC provided comparable signal intensities, as well as signal-to-noise values, to UHPLC. For the remaining two peptides, either the ratio is close to one (in the case of liraglutide) or simply UHPSFC did not provide the same MS sensitivity as UHPLC does (in the example of eptifibatide). Interestingly, the ionic species generated by the two chromatographic techniques were not always similar (Fig. 9). This was also observed in the previous section, as all impurities detected under UHPSFC has a lower m/z ratio than in UHPLC. Indeed, it appeared that UHPSFC was able to better protonate peptides, especially those with a relatively long chain (liraglutide and glucagon) compared to UHPLC, indicating a higher charge state of the ions. This phenomenon was already observed by Wang and Olesik [38], describing how the employment of mobile phases containing liquified CO_2 provided increased charged states and narrower charge state distributions. The authors claimed that the addition of liquified CO_2 mainly improved the desolvation process in the ESI ionization chamber.

3.3.3. Transferability of the UHPSFC method for peptides to preparative scale

We next focused our efforts on a semipreparative purification of a cyclic peptide API. This mixture was subjected to automated SFC column screening [36] on eight different stationary phase columns with gradient elution using MSA-rich modifiers (Fig. 10a). Several columns were found to effectively separate the two components in this reaction showing excellent peak shape and acceptable resolution (2-PIC, DEA and 4-EP). A straightforward optimization to isocratic elution: 35% MeOH/H_2O 95:5 *v/v* + 8 mM MSA/ 65% CO2 on a Waters Torus 2-PIC (30.0 mm x 250 mm, 5 µm) column at a flow rate of 140 mL/min enabled baseline resolution at the semipreparative scale. This procedure facilitated a rapid delivery of 84 mg of peptide (purity > 98%, yield > 95%) by five x 1 mL stacked injections of 20 mg/mL peptide mixture (purity ≈ 69%) in less than 15 min total runtime (Fig. 10b). This serves to illustrate the power of modern SFC technologies and the practical use

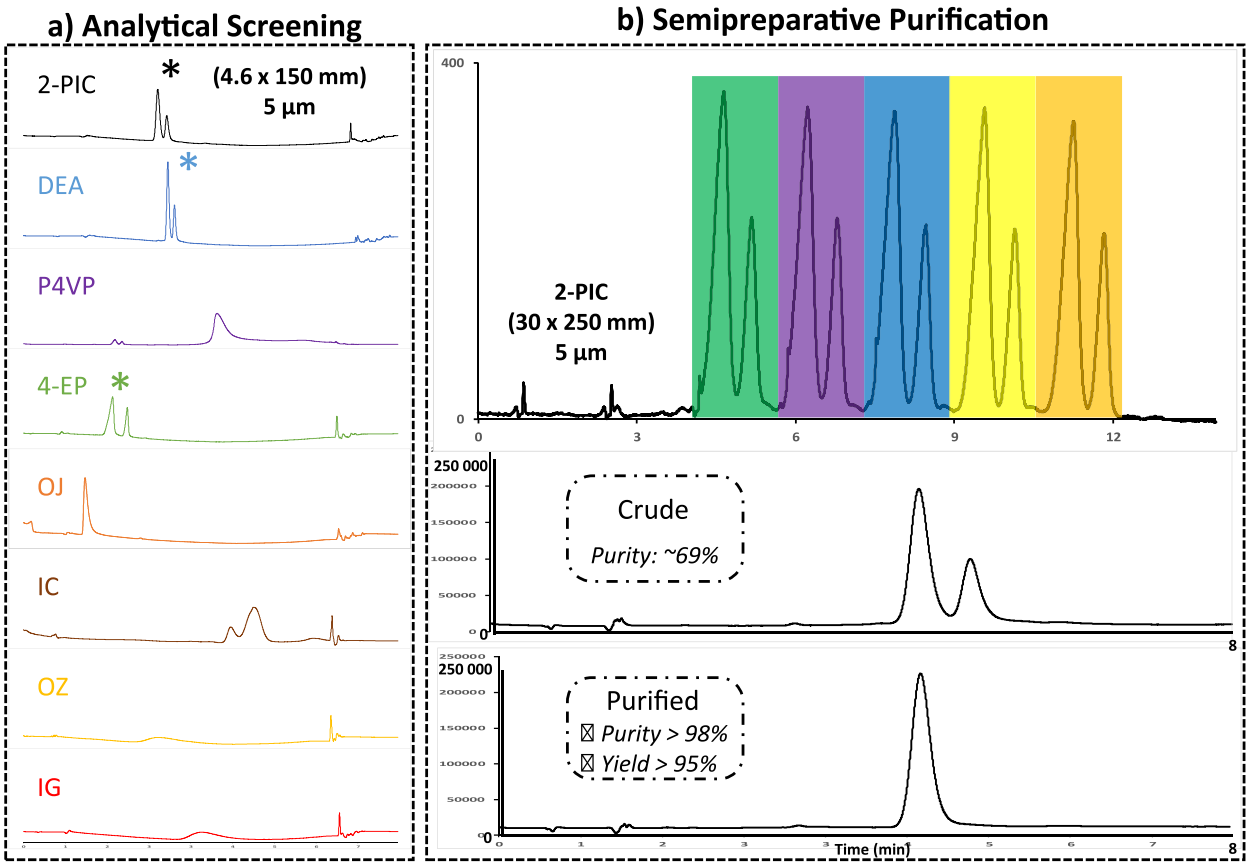

Fig. 10. a) Automated column screening of cyclic peptide using the mobile phase conditions in the Experimental section. b) Semipreparative purification of the cyclic peptide on the 2-PIC column using the conditions listed in the Experimental section (top), the analysis of the sample before purification (middle) and after purification (bottom).

of MSA-rich modifiers in pharmaceutical setting at both analytical and preparative scale.

4. Conclusions

In this work, the possibilities offered by UHPSFC coupled to UV and MS detectors, was evaluated for a series of synthetic and commercially available peptides. A systematic comparison with UHPLC was performed to draw the advantages and limitations of UHPSFC for this kind of analytes. At first, the choice of the stationary phase, as well as an optimization of the mobile phase conditions were achieved for UHPSFC. The combination between a positively charged stationary phase with the addition of an acidic additive in the mobile phase was found to be the one offering the best performance for peptide analysis. Later, the evaluation of a novel additive, methanesulfonic acid (MSA), was carried out and results were compared to the more commonly used trifluoroacetic acid (TFA). MSA demonstrated to provide significantly better chromatographic performance against TFA, at a lower concentration in the mobile phase (8 mM vs 15 mM).

In the second part of this study, the selectivity achieved in UHPSFC was discussed, with a systematic comparison with UHPLC conditions. UHPSFC provided a different separation of peptides according to their isoelectric points vs. UHPLC. Furthermore, UHPSFC allowed a very good discrimination between peptides with different amino acidic sequence lengths, while no such relationship was demonstrated with UHPLC.

In the third part of this work, some applications of UHPSFC in peptide analysis were evaluated and systematically compared to UHPLC, both hyphenated to a MS detector. To this purpose, a set of six commercially available peptides, of which three possessing a linear structure and three with a cyclic one, were employed. Different stressing procedures were employed on each peptide, exposing them to either acidic, basic or oxidative stress. Results showed that UHPSFC gave comparable, if not sometimes even better, performance to those observed with UHPLC. Regarding the MS sensitivities achieved in UHPSFC, it was seen that they were comparable to those observed under UHPLC conditions. Finally, the transferability of the developed analytical method to a semi-preparative level was considered, and the semi-preparative SFC method shows excellent performance in terms of yield and purity for a Merck cyclic peptide.

All these results demonstrated that UHPSFC is a viable alternative for the analysis of highly polar compounds with high molecular weight such as peptides, utilizing a gradient reaching high percentages of co-solvent. Furthermore, UHPSFC has shown once more its orthogonality against UHPLC, fueling even more its utility in analytical laboratories.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Gioacchino Luca Losacco: Writing - original draft, Methodology, Investigation. **Jimmy Oliviera DaSilva:** Investigation, Writing - review & editing. **Jinchu Liu:** Investigation, Writing - review &

editing. **Erik L. Regalado:** Supervision, Writing - review & editing, Resources. **Jean-Luc Veuthey:** Supervision, Writing - review & editing. **Davy Guillarme:** Supervision, Writing - review & editing, Project administration.

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Chapter III – Conclusions and perspectives

In the present thesis work, various aspects of UHPSFC have been explored, together with innovative applications in fields where UHPSFC was scarcely employed. Initially, investigations have been made in assessing the actual reproducibility that an UHPSFC method can offer in routine environments. Therefore, its implementation in the anti-doping workflow for routine analyses has been evaluated. In previous thesis works, it was demonstrated how UHPSFC represents an interesting alternative to RPLC in this field, but an evaluation of its behavior in routine laboratories was needed. Therefore, the stability of the retention times of 51 doping agents, in neat solutions as well as spiked in diluted urine, has been first assessed. A total period of four months, in which the analyses were repeated every 2-3 weeks, was needed to gather the necessary data. Three different UHPSFC columns were tested, since the column screening is an important part of the method development process in UHPSFC. The results have demonstrated how two of the three columns (BEH silica and Torus 2-PIC) provide a good method robustness, while the third (HSS C18 SB) generates an unacceptable variability. To explain these results, it was hypothesized that the systematic addition of water in the mobile phase, although at limited concentrations, was able to greatly limit the SEF phenomenon on the surface of the silica particles. The BEH silica column was, therefore, able to ensure a good robustness, while with the HSS C18 SB it is believed that the water molecules cannot limit the SEF as they are not able to properly reach the surface of the silica particle, due to the presence of the apolar C18 selector. On the Torus 2-PIC, however, the combined use of water together with a new generation of silica particles on which the presence of free silanols has been greatly reduced contributed to the overall best performance of this stationary phase. The intercolumn variability was also taken into consideration, by confronting same stationary phases belonging to different production lots. No major sources of variability were found related to that aspect. Finally, a comparison against an UHPLC method regularly used in routine anti-doping analyses illustrated how UHPSFC, using the Torus 2-PIC column, is extremely close to UHPLC in terms of method reproducibility when biological samples are injected.

Subsequently, the developed UHPSFC-MS/MS method was tested in terms of interlaboratory variability (reproducibility). Due to the historically poor performance in the context of method validation with SFC, this aspect requires a throughout investigation to establish if UHPSFC can now satisfy the robustness criteria not only in one laboratory, but more importantly when the same method is employed in different laboratories from several countries. Therefore, four laboratories present in Europe have participated in this interlaboratory study, in which they analyzed a library of 21 doping agents spiked in diluted urine at two concentration levels, as well as performing the identification and estimation of concentration on seven blind urines. In each of the blind urine, between zero to two doping agents (available in the previous library)

were present but none of the laboratory knew which analyte was present in each sample. The results obtained have been extremely promising since the analytical method demonstrated a low variability for the vast majority of the doping agents (19 out of 21 with an RSD below 3.0%). More importantly, each laboratory was capable to identify the right compound(s) present in the seven blind urines, as well as providing coherent estimations of their concentration levels, respecting the minimum required performance level (MRPL) established by the WADA for each class of doping agent.

After an evaluation of the robustness of UHPSFC, the focus moved towards providing an overview of some theoretical considerations in UHPSFC when a large amount of co-solvent is used. Our work highlights how the use of elevated levels of the organic modifier (> 50-60%) unlocks the analysis of more challenging samples in UHPSFC, such as nucleosides and amino acids. This type of approach has been defined as Unified Chromatography by some researchers. However, little is known about the possible effects of some parameters that might affect the quality of UC-type separations in UHPSFC. More specifically, in this work the impact of different column temperatures was taken into consideration. This parameter can become quite important in UHPSFC, as it can impact the mobile phase density, and thus its elution strength. However, an update of its role was needed as modern applications in UHPSFC are using very high percentages of co-solvent, thus shifting the state of the mobile phase from super/subcritical fluid to a liquid-like or, in few cases, even liquid. At first, the kinetic performance at five temperatures was evaluated (-5°C, 5°C, 15°C, 40°C, 80°C). The van Deemter plots indicated that low temperatures appear as the best choice with a mobile phase rich in $scCO_2$. However, as the liquid component increases, higher temperatures become more favorable. Secondly, thermodynamic considerations have also been covered. The van't Hoff plots collected at the different temperatures, using a set of four analytes eluting at different mobile phase ratios, illustrate the presence of three types of retention behaviors: those eluting with a low percentage of co-solvent followed a typical SFC-like behavior (decrease in retention with lower temperatures), while compounds eluting at high percentages (>70%) showed a LC-like pattern (decrease in retention with higher temperatures). However, analytes eluting in the range 15-60% of modifier had a U-shaped behavior, resulting from the mixing of both SFC- and LC-like trends when temperature changed. Applications in which the use of either very low (5°C and -5°C) or high temperatures (80°C) have been developed for a series of different compounds, illustrate how unusual temperatures may be beneficial, in some cases, to obtain the best results.

In the second part of this thesis work, the use of UHPSFC in different fields has been evaluated. Its applicability in metabolomics was first assessed. Metabolomics, defined as the identification

and quantification of metabolites in living organisms, is a field in which there is a strong interest in developing innovative approaches which would increase the overall performance (analyte coverage, sensitivity…). UHPSFC represents a valid solution as it offers a good orthogonality to LC techniques, mainly RPLC and HILIC, however its compatibility with the most polar metabolites was often questioned. In the first article, the development and optimization of a UHPSFC method was carried out, using a restricted set of 57 metabolites belonging to different classes (i.e. amino acids, nucleosides, lipids, steroids, etc.). Using a UC-type gradient, in combination with a polar column (Poroshell HILIC), it was possible to generate the highest detectability rate, eluting metabolites with log P ranging from 10 to -5. Specifically, thanks to the use of water in the mobile phase, as well as the combination of different salts (ammonium formate and ammonium fluoride) at relatively high concentrations (50 and 1 mM, respectively), the best chromatographic performance was obtained. In the second paper of the series, a scale up in the metabolite's number was done, from 57 to 597, using the Sigma Metabolite Library. UHPSFC reached a total detection rate of 66%, demonstrating excellent performance not only for apolar compounds (lipids, steroids), but also for highly polar metabolites (amino acids, sugars, and nucleosides). However, the developed method gave poor results with compounds possessing either one or more phosphoric groups in their structure (e.g. nucleotides). In the same work, the impact of biological matrices, such as urine and plasma, was then studied, using a restricted library of 51 metabolites. The ME generated at the MS ionization source was, first assessed: with both matrices, UHPSFC did not suffer from important ME, with more than 70% of compounds showing either no or limited variations of their signal intensities. Differences in the ME between urine and plasma were also observed. Moreover, in the majority of MS/MS spectra, no interferences deriving from compounds belonging to the matrix were recorded. Finally, the UHPSFC method yielded satisfactory results in terms of retention times stability, using both matrices over a period of three weeks.

Finally, a last application of UHPSFC involved the analysis of peptides and related impurities. In this area, the gold standard method is RPLC, however UHPSFC attracted interest because of its well-documented complementarity to the former. Initially, the method development stage indicated that the combination of a stationary phase with basic properties, such as the Torus 2-PIC, with an acidic additive in the mobile phase was the best method for a series of six synthetic peptides. A further study on the additive resulted in the use of a strong organic acid, methanesulfonic acid (MSA), instead of trifluoroacetic acid as it is capable to create a more acidic environment, thus boosting the chromatographic performance with sharper and more symmetric peaks at reduced concentrations. The comparison of the developed UHPSFC method with RPLC gave some interesting results. Both methods were able to separate a series of six synthetic peptides with different pI values, giving a very different elution profile.

Furthermore, when a second series of synthetic peptides with identical pI value but increasing chain length was tested, UHPSFC was clearly superior to RPLC, being able to efficiently separate peptides with six to twenty-one amino acids in their chain; RPLC, on the other hand, was not able to discriminate between them. The orthogonality between the two chromatographic techniques was highlighted also by the analysis of six commercial peptides that were exposed to different stressing procedures. Finally, a scale-up to a semi-preparative level of the analytical UHPSFC method provided excellent results in terms of yield and purity with a cyclic peptide.

Overall, this thesis work confirms the flexibility and versatility of UHPSFC for a wide range of compounds. This technique performs well with apolar and, more importantly, with highly polar analytes. Even when the complexity of the samples was increased, UHPSFC was still able to give interesting results, such as for peptides. Moreover, the assessment of its reproducibility using conditions such as those employed in routine laboratories, where several regulations need to be fulfilled, showed how it has closed down the distance that used to separate UHPSFC from the highly robust UHPLC. However, some issues related to the technique and its instrumentation were encountered. An unacceptable variability of the retention times for early-eluting doping agents was recorded during the interlaboratory analysis, most probably due to differences in the handling of the mobile phase's density, although the same chromatographic system was utilized by all participating laboratories (Waters Acquity UPC^2). Secondly, UHPSFC does not always guarantee the same sensitivity levels as in UHPLC; this was demonstrated during the analysis of peptide's impurities using MS as the detector. The use of columns with higher volume in UHPSFC (generally with an internal diameter of 3.0 mm) than those in UHPLC (internal diameter of 2.1 mm) certainly contributes to the addition of a dilution factor which negatively impact the sensitivities achievable with the former technique. Furthermore, the increase in the co-solvent amounts up to 100% of the mobile phase's composition has further highlighted that the current generation of UHPSFC systems suffers from a too restricted pressure range (upper limits fixed at 400-660 bar), as well as from a too significant system volume which inevitably contributed to the widening of peaks, symptom of a poor kinetic efficiency. Finally, while good performance were obtained with highly polar compounds, poor results have been found with phosphorylated analytes. To further improve this technique, the design of the chromatographic system should be improved, with the aim to increase the upper pressure limit as well as reducing the extra-column volume. Finally, an attempt in using bioinert materials (PEEK coated, titanium, MP35N, hybrid surfaces…), either at the column level or on the chromatographic instrumentation, should be made, with the scope to enhance the detectability of samples possessing phosphorylated groups.

Nonetheless, UHPSFC has highlighted its great potential as a technique able to analyze compounds with an impressive range of polarities within a single analytical run, using the same instrument and the same composition of the mobile phase, which is rarely seen in UHPLC. Furthermore, UHPSFC is considered a technique with a reduced environmental impact than UHPLC, since the mobile phase is mainly composed in CO_2 which is less toxic than other organic solvent generally employed in UHPLC such as acetonitrile or hexane. In addition, it can also help in the cost reduction for one analysis, due to low prices for CO_2 and methanol, the latter being also used in relatively limited quantities. Our idea of the perspectives for UHPSFC revolves on the analysis of samples with an increased complexity and higher molecular weight. Indeed, until now UHPSFC has been mostly utilized for small molecules, with a molecular weight of less than 1000 Da. However, in the pharmaceutical industry, there has been a gradual shift towards the development of larger molecules, such as peptides, monoclonal antibodies, and oligonucleotides. While intact proteins (MW > 50 000 Da) can remain extremely challenging, samples with a MW ranging from 1000 to 10 000 Da should become the next target for UHPSFC, such as larger peptides, fragments of proteins, oligonucleotides as well as oligosaccharides (e.g. glycans). Additionally, developments in understanding and improving the method transfer from analytical to preparative scale should be of interest, to improve the performance, reliability, and easiness of such procedure.

Chapter IV – Appendices

IV.1 Enantiomeric methadone quantification on real post-mortem dried matrix spot samples: comparison of LC and SFC to MS

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Enantiomeric methadone quantitation on real post-mortem dried matrix spots samples: Comparison of liquid chromatography and supercritical fluid chromatography coupled to mass spectrometry

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ABSTRACT

This study describes two bioanalytical methods for the quantitation of the two methadone enantiomers in dried matrix spots using high performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) and high performance supercritical chromatography tandem mass spectrometry (HPSFC-MS/MS). Dried matrix spots were obtained by spotting 10 μL of each sample fluid on a Whatman paper. Methadone and its main metabolite, EDDP, were extracted with 100 μL methanol and subsequently injected into the LC-MS/MS and SFC-MS/MS systems. Enantiomeric separation was achieved with AGP-column for the LC conditions and with Chiralpak IH-3 in SFC. The two methods were fully validated and 93 post-mortem samples were analysed with both analytical methods. Results from validation parameters and results obtained for all post-mortem samples were compared with a significant spearman correlation of $r_s = 0.9978$ for R-methadone and $r_s = 0.9981$ for S-methadone. The LC method provided better results in terms of uncertainty, retention factor and resolution, whereas SFC provides better sensitivity, with lower LOD. Median R-/S-methadone ratio in peripheral blood was found equal to 1.60 (N = 32), varying from 0.79 to 4.23. The reported values were in good agreement with previously published results.

Based on the results obtained here, SFC-MS/MS can be considered a reliable alternative to the widely used LC-MS/MS for the quantitation of methadone enantiomers in bioanalysis and should be evaluated for other bioanalytical methods. Both methods can be easily and quickly used in toxicological routine analysis for the methadone quantitation in human fluids matrices, even if considering that the polysaccharide coated column IH-3 used in SFC does not allow the enantiomeric EDDP separation.

1. Introduction

Methadone is a μ-opioid receptor agonist similar to morphine and is administered for chronic pain and opioid related dependence [1,2]. R-methadone has a higher μ and δ opioid receptor activation and a greater analgesic activity compared to the S-methadone [2]. Methadone is also shown to increase QT dispersion as well as QT interval [3,4] mainly through the S-methadone enantiomer, because of its 3.5-fold more potent hERG channel blockage compared to the R-methadone [5]. In this context, even if the use of R-methadone enantiomer is suggested [6], methadone is still clinically administered in the racemic form. Due to the high interindividual variability in R-/S-methadone stereoselective

Abbreviations: ACN, acetonitrile; AGP, alpha 1-acid glycoprotein; CB, cardiac blood; DBS, dried blood spot; DMS, dried matrix spot; EDDP, 2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; ESI, electrospray ionization; EtOH, ethanol; LC, liquid chromatography; LLE, liquid liquid extraction; MeOH, methanol; MS/MS, tandem mass spectrometry; MTD, methadone; PB, peripheral blood; PF, pericardial fluid; PrOH, propanol; QC, quality control; RT, retention time; SPE, solid phase extraction; SFC, supercritical fluid chromatography; S/N, signal to noise.

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metabolism [7], ratio between R-methadone and S-methadone in blood is shown to be significantly different between individuals and vary from 1 to 4 [8,9].

The first article describing the analytical enantiomeric separation of methadone in plasma has been published in 1991 by Beck et al. [10] using LC with an α1-acid glycoprotein (AGP) column coupled with an UV detector. Since then, several methods mainly using LC-UV have been proposed [11–14] with an off-line sample preparation performed by LLE or SPE. More recently, enantiomeric methadone separation was carried out with LC coupled with MS to quantify methadone enantiomers in human post-mortem samples, including fluids and tissues [8,9].

For almost 50 years, dried blood spots (DBS) have been used for collection, analyses and long-term storage of blood samples. In the last two decades, DBS also became a more discussed matrix in forensic toxicology analysis. For instance, Odoardi et al., [15] developed an LC-MS/MS method to analyse drugs of abuse using a DBS matrix. Recently, Metzger et al. [16], have quantified R- and S-methadone in DBS matrix from real human cases using an LC-MS/MS without a full description of the method validation results. Compared to the LC platforms, dried matrix spots have been scarcely employed in conjunction to SFC technique until now, and only a few studies were published in the literature [17,18]. Although chiral SFC is largely used in the pharmaceutical industry, its application in forensic analysis is limited [19,20], since LC and GC remain the most commonly used techniques for chiral separation [21].

Several studies comparing SFC and LC have been published [22–26], some of them evaluating SFC-MS/MS and LC-MS/MS performance in the field of bioanalysis [22,23,27]. For instance Borovcova et al. [27] described a systematic comparison between validation parameters results from the two different instrumental techniques used for the determination and the quantitation of 15 new psychoactive drugs. Hoke et al. [22] compared validation results and human plasma samples results obtained separating the ketoprofen enantiomers with the same column with both LC-MS/MS and SFC-MS/MS.

The present study describes two analytical methods for the separation and quantitation of methadone enantiomers, using both SFC-MS/MS and LC-MS/MS. These two bioanalytical methods were fully validated for chiral methadone quantitation and a series of 93 DMS obtained from real post-mortem cases were then successfully analysed with both methods.

2. Materials and methods

2.1. Reagents and standards

Reference solutions of R-/S-MTD, R-/S-EDDP at 1 mg/mL in methanol and R-/S-MTD-D9, R-/S-EDDP-D3 at 0.1 mg/mL in methanol were obtained from Lipomed (Lipomed, Switzerland). R-methadone was obtained from L-Polamidon medicament (Mundipharma Medical Company, Basel). Methanol, acetonitrile and isopropanol were obtained from Carlo Erba (Carlo Erba, Italy). Formic acid 98–100% LC-MS grade and ammonium acetate were purchased from Sigma (Sigma, Germany). H_2O was obtained from Milli-Q system from Millipore. All solvents and inorganic chemicals were of analytical grade. Whatman 903 Paper Saver Snap Apart cards used for dried matrix spots were obtained from Whatman (Whatman, United Kingdom). Pressurized carbon dioxide (CO2, 99.99%) was purchased from Carbagas (Lausanne, Switzerland) and was employed for the SFC measurements.

2.2. Preparation of calibration curves

Methadone and EDDP solutions were used to prepare calibration standards in blank bovine whole blood with concentrations of 20, 50, 100, 200, 500, 1000, 2000, 5000 ng/mL for each racemate. 10 µL of each calibration sample was spotted to the Whatman paper and dried for at least 3 h at room temperature before extraction.

2.3. Postmortem cases

Autopsy cases were selected in which methadone was revealed in femoral blood during toxicological routine analysis from January 2016 to July 2020. When possible, the following matrices were collected during autopsy for the present study: femoral whole blood, cardiac whole blood, vitreous humour and pericardial blood. All samples were stored at −20 °C until analysis. Femoral blood, cardiac blood and vitreous humour were stabilized with sodium fluoride. A total of 93 post-mortem samples from 35 post-mortem cases related to the methadone were analysed: 32 peripheral bloods, 29 cardiac bloods, 23 vitreous humour and 9 pericardial fluids.

2.4. Sample preparation

The same samples were used for LC-MS/MS and SFC-MS/MS analyses. 10 µL of calibration, QC and human post-mortem samples (i.e. whole blood, cardiac blood, vitreous humour, pericardial fluid) were added to the Whatman paper and dried for at least 3 h at room temperature prior extraction. Dried matrix spots were cut and added to an Eppendorf with 100 µL internal standard mix in methanol (40 ng/mL Methadone-d9 and 10 ng/mL EDDP-d3). Eppendorf were vortexed for 30 s and incubated at RT for 30 min. Then, extracts were transferred into vials and a volume of 0.5 µL was injected in both LC and SFC conditions.

2.5. Instrumentation and analytical method

2.5.1. LC-MS/MS

LC separations were performed on an Acquity UPLC *I-class* system (Waters, Milford MA, USA) composed of a binary solvent delivery pump, an autosampler with flow through needle (SM-FTN) injection system and a column oven equipped with an active preheater. The autosampler temperature was fixed at 15 °C and the column was heated at 25 °C. The Acquity separation module was coupled to a Xevo TQ-XS mass detector equipped with an ESI interface (Waters, Milford MA, USA). Chromatographic separation was achieved using AGP stationary phase (Chiral Technologies, France) (100 × 2.1 mm i.d., 5 µm particle size) with an AGP guard column (10 × 2.0 mm i.d., 5 µm particle size) at 25 °C. The mobile phase consisted of aqueous ammonium acetate 10 mM pH 5.8 (A) and isopropanol (B). The following gradient elution was used (runtime 14 min), starting with 6% B for 8 min, increased to 8% B at 8.10 min, held to 11 min, increased to 20% B at 11.20 min, held to 12 min, and returned to initial conditions of 6% B at 12.10 min and maintained until 14 min. The flow rate was assessed at 0.3 mL/min.

The ESI source was operated in the positive mode with the following conditions: source temperature and desolvation gas (nitrogen) temperature were set at 150 °C and 650 °C, respectively, the gas flow was delivered at 1000 L/h and the capillary voltage was set at 1.0 kV. Product ions were obtained by collision-induced fragmentation in the multiple reaction monitoring (MRM) mode. MRM transitions and conditions for measurement of methadone were: 310 m/z > 105 m/z, 310 m/z > 223 m/z, 310 m/z > 265 m/z (quantifier); cone voltage 35 V, collision energy 26 eV, 22 eV and 14 eV, respectively. Methadone-d9 are: 319 m/z > 105 m/z (quantifier), 319 m/z > 268 m/z; cone voltage 35 V, collision energy 30 eV and 15 eV, respectively. EDDP are: 278 m/z > 186 m/z, 278 m/z > 219 m/z, 278 m/z > 249 m/z (quantifier), cone voltage 30 V, collision energy 32 eV, 38 eV and 22 eV, respectively. EDDP-d3 are: 281 m/z > 234 m/z (quantifier), 281 m/z > 249 m/z; cone voltage 30 V, collision energy 30 eV and 24 eV, respectively. Waters MassLynx software Version 4.2 was used for instrument control and quantitation.

2.5.2. SFC-MS/MS

The analyses have been performed using on a Waters Acquity UPC^2 system (Waters, Milford, MA, USA) equipped with a Binary Solvent Manager delivery pump, a Sample Manager autosampler which included

a 10 μL loop for partial loop injection, a column oven with active pre-heater, a PDA detector with an 8.4 μL flow-cell and a two-step (active and passive) backpressure regulator (pre-BPR). The chromatographic system was hyphenated to a Waters Xevo TQ-S mass detector equipped with an ESI interface via a double-T splitter interface from Waters (Waters, Milford MA, USA) [28]. Chromatographic separation was achieved using a Chiralpak IH-3 stationary phase (Chiral Technologies, France) (150 mm length × 3.0 i.d., 3 μm particle size). Elution solvents consisted of CO_2 (A) and Methanol:H_2O (98:2, v:v) containing ammonium acetate 20 mM (B). The following elution gradient was used (runtime 14 min), from 0 to 9 min 10% B, increased to 40% B at 9.5 min, held to 12 min, changed to 10% B at 12.20 min and maintained to the initial conditions till 14 min. The flow rate was 0.4 mL/min and the injected volume was 0.5 μL. The automated backpressure regulator (ABPR) was set at 150 bar, with the make-up flow set up at 0.5 mL/min and the column temperature was set at 30 °C.

The electrospray source was operated in the positive ionization mode (ESI+). The source temperature and desolvation gas (nitrogen) temperature were set at 150 °C and 450 °C, respectively. The flow gas was delivered at rate of 1000 L/h. The capillary voltage was set at 1.0 kV. MRM transitions and conditions for measurement of methadone are: 310 m/z > 77 m/z, 310 m/z > 105 m/z, 310 m/z > 265 m/z (quantifier); cone voltage 25 V, collision energy 48 eV, 29 eV and 15 eV respectively. Methadone-d9 are: 319 m/z > 105 m/z, 319 m/z > 268 m/z (quantifier); cone voltage 25 V, collision energy 29 eV and 15 eV respectively. EDDP are: 278 m/z > 186 m/z, 278 m/z > 234 m/z, 278 m/z > 249 m/z (quantifier), cone voltage 25 V, collision energy 35 eV, 30 eV and 25 eV respectively. EDDP are: 281 m/z > 234 m/z (quantifier), 281 m/z > 249 m/z; cone voltage 30 V, collision energy 30 eV and 23 eV respectively. Waters Mass-Lynx system software Version 4.2 was used for instrument control and quantitation.

2.6. Method validation

Validation for both LC-MS/MS and SFC-MS/MS methods was performed in agreement with the document: "Guideline on bioanalytical method validation" published by the European Medicines Agency (2016). The following parameters were assessed: calibration model, selectivity, specificity, accuracy, precision, carry-over, interferences, ionization suppression/enhancement, recovery, limit of detection (LOD), limit of quantitation (LOQ), uncertainty and stability.

Accuracy and precision were determined for each QCs (quality controls for enantiomer: LLOQ 10 ng/mL, low 30 ng/mL, medium: 1000 ng/mL and high 2000 ng/mL) in five replicates and in five independent analytical runs. Sensitivity was determined for the LLOQ in in six replicates and in five independent runs. Selectivity and specificity were determined by injecting 10 different human blood samples, which were fortified at the QC LLOQ, and injecting six different human blood samples containing the following drug groups: benzodiazepines, THC, cocaine, opioids, LSD, antidepressant and neuroleptics. Carry-over was evaluated in triplicate following injection of the 3xULOQ (ULOQ: upper level of quantitation) calibration standard. Recovery was assessed by comparing pre-spike samples with post-spike samples in triplicate for three different QCs (low, medium, high). Matrix effect was determined by comparing post-spike samples in matrix, with the post-spike samples without matrix in triplicate for three different QCs (low, medium, high). Limit of quantitation (LOQ) was defined to be the first calibration point and limit of detection was evaluated visually for signal-to-noise ratio S/N = 3. Stability of methadone and EDDP on DBS matrix was assessed using 3 different QCs levels (low, medium, high) stored for 5 months and analysed in triplicate using a freshly prepared calibration curve.

3. Results

3.1. Chromatographic enantiomeric separations

R-/S-methadone and R-/S-EDDP were resolved using LC-MS/MS platform equipped with the AGP column (see Fig. 1). This was in agreement with the results published by Beck et al. [10]. The widths of the peaks at baseline were the following: R-methadone 1.2 min, S-methadone 1.4 min, R-EDDP 0.7 min, S-EDDP 0.9 min.

To achieve a suitable separation in SFC, various mixtures of organic modifiers and CO2 were initially tested under isocratic conditions for the analysis of methadone only, including CO_2/MeOH (80:20), CO_2/EtOH (80:20), CO_2/2-PrOH (80:20) and CO_2/ACN (80:20). The proportions of CO_2 and organic modifiers in the mobile phase were further adjusted to obtain retention factors between 1.5 and 15 for methadone and EDDP and gradient elution (up to 40% MeOH) was used. Finally, some additives (2% water and ammonium acetate 20 mM) were added to the mobile phase to improve peak shapes for the basic drug and metabolite. In SFC, R-methadone was injected alone to know which chromatographic peak corresponds to which enantiomer. Fig. 2 shows the corresponding methadone enantiomeric separation, and the elution order of the two methadone enantiomers was the same in both LC and SFC. On the other hand, chiral separation of R-/S-EDDP was not achieved, due to the lower chiral selectivity of the Chiralpak IH-3 column compared to the AGP column. The peak widths at baseline were as follow: R-methadone 0.45 min, S-methadone 0.40 min, R-/S-EDDP 0.18 min.

Retention factor (k'), selectivity (α), column efficiency (N) and resolution (R_S) were calculated for both analytical separations and results are presented in Table 1. Both chromatographic methods showed a satisfactory enantiomeric methadone separation, with a greater differentiation obtained in LC. In details, selectivity (α) are better in LC compared to that obtained in SFC for both methadone and EDDP. However, column efficiency (N) is much higher in SFC compared to LC for both compounds. In the end, it appears that the resolution (R_S) of 3.61 obtained in LC for the methadone enantiomers was quite higher than the one obtained in SFC (R_S = 1.80). When comparing the two analytical conditions, the enantiomeric separation in LC was achieved using a protein coated stationary phase, which has shorter lifetime, a maximum tolerable of 40–50% organic concentration in mobile phase, a long-term storage recommended in the fridge and, unfortunately, a lower repeatability between batches. On the other hand, the polysaccharide coated column used in SFC allows a much better repeatability between batches, a long-term storage at room temperature, possibility to increase organic concentration in mobile phase and longer lifetime compared to the AGP column [29].

Both, LC-MS/MS and SFC-MS/MS methods offer the same runtime of 14 min. Figs. 3 and 4 show the blank DBS and the lowest calibration point in DBS for both analytical methods using LC-MS/MS and SFC-MS/MS, respectively. Background noise in LC was higher compared to that observed in SFC.

3.2. Methods validation results

Comparison between methods validation parameters obtained in LC-MS/MS and SFC-MS/MS are shown in Tables 2, 3 and 4.

Mean determination coefficients of the calibration curves were calculated for five different analytical runs. Mean values for R-methadone in LC-MS/MS and SFC-MS/MS (r^2 value was equal to 0.9992) was the same (see Table 1). Mean correlation coefficient for S-methadone in LC-MS/MS and SFC-MS/MS were also totally comparable (R^2 of 0.9992 and 0.9991 in LC-MS/MS and SFC-MS/MS, respectively) (see Table 2). Results for accuracy and precision of R- and S- methadone enantiomers were obtained by analysing 4 QCs levels (LLOQ: 10 ng/mL, low: 30 ng/mL, med: 1000 ng/mL and high: 2000 ng/mL) in quintuplicate. Mean QCs accuracy for R- and S-methadone in LC was 3.93 and 3.85%,

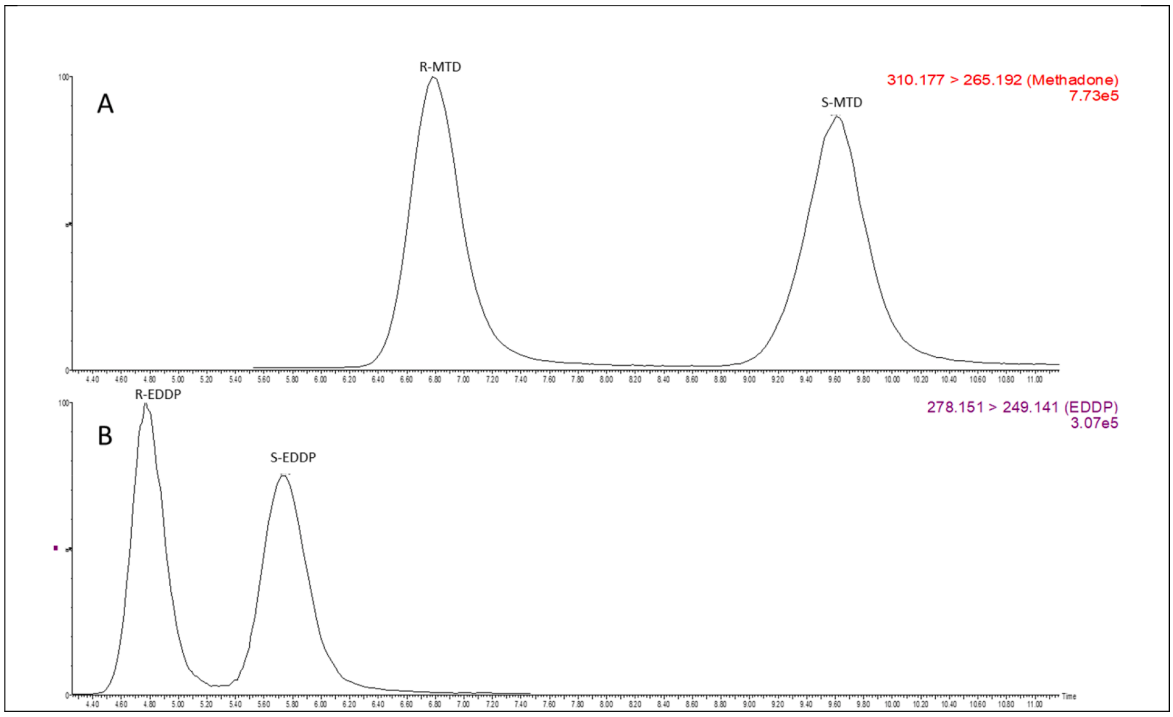

Fig. 1. Chiral LC separation using AGP column. Standards racemic mixtures were analysed at a concentration of 60 ng/mL. (A): R-methadone (RT: 6.78 min), S-methadone (RT: 9.61); (B): R-EDDP (RT: 4.77 min), S-EDDP (RT: 5.75 min).

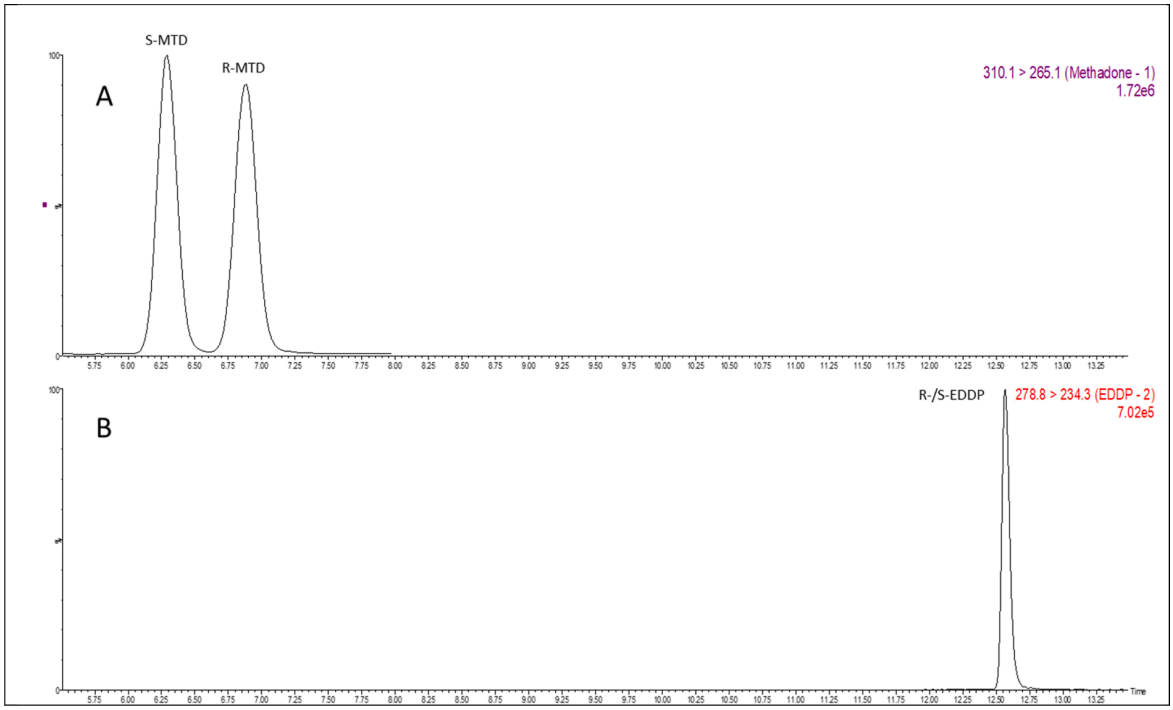

Fig. 2. Chiral SFC separation using Chiralpak IH-3 column. Standards racemic mixtures were analysed at a concentration of 60 ng/mL. (A): R-methadone (RT: 6.29 min), S-methadone (RT: 6.89); (B): R-/S-EDDP (RT: 12.57 min).

respectively. Mean QCs accuracy for R- and S-methadone in SFC was 2.78 and 2.70%, respectively. Highest bias level calculated in LC was 6.9% (R-methadone QC LLOQ), whereas in SFC it was 5.5% (S-methadone QC high). Mean precision (between run) for R- and S-methadone in LC was 3.28 and 4.20%, respectively. Mean precision (between run) for R- and S-methadone in SFC was 6.20 and 6.35%, respectively. The worst precision level calculated in LC was 7.3% (within run, R-methadone QC LLOQ), while in SFC, it was 10.5% (between run, R-methadone QC LLOQ). Methadone recovery using a simple methanol extraction was close to 100%. The matrix effect using LC-MS/MS analysis was

Table 1
Results comparison between UHPSFC and UHPLC for the main chromatographic parameters.

		Retention time (RT)	Retention factor (k)	Selectivity (α)	Resolution (Rs)
LC					
	t_0	0.74			
R-MTD	t_{R1}	6.78	8.16		
S-MTD	t_{R2}	9.61	11.99	1.47	3.61
R-EDDP	t_{R1}	4.77	5.45		
S-EDDP	t_{R2}	5.75	6.77	1.24	1.81
SFC					
	t_0	2.02			
R-MTD	t_{R1}	6.29	2.12		
S-MTD	t_{R2}	6.89	2.41	1.14	1.80
R-/S-EDDP	t_{R1}	12.57	5.23		

negligible and a slight ion enhancement was observed in SFC-MS/MS. According to the validation design, LOQ in LC and SFC was defined at the first calibrator at 10 ng/mL for both methadone enantiomers. LOD (Calculated for S/N = 3) in LC was 2.5 ng/mL for both methadone enantiomers, whereas it was equal to 0.5 ng/mL in SFC. Sensitivity in SFC was about 5-times better than in LC for methadone enantiomers. No interferences were observed after the injection of six different human blood samples containing benzodiazepines, cocaine, amphetamines, opioids, antidepressants, neuroleptics and cannabinoids. Uncertainty was calculated using guidelines from ISO/IEC [30] and defined to be 2 times the highest SD. Uncertainty for R- and S- methadone quantitation in LC-MS/MS was lower compared to that calculated in SFC-MS/MS. In the case of methadone metabolite (EDDP), mean correlation coefficients of the calibration curves were calculated for five different analytical runs. The average values for R-EDDP and S-EDDP in LC and for the racemic R-/S-EDDP in SFC were the same, with an R^2 value of 0.9995 (see Table 3). EDDP recovery using a simple methanol extraction was

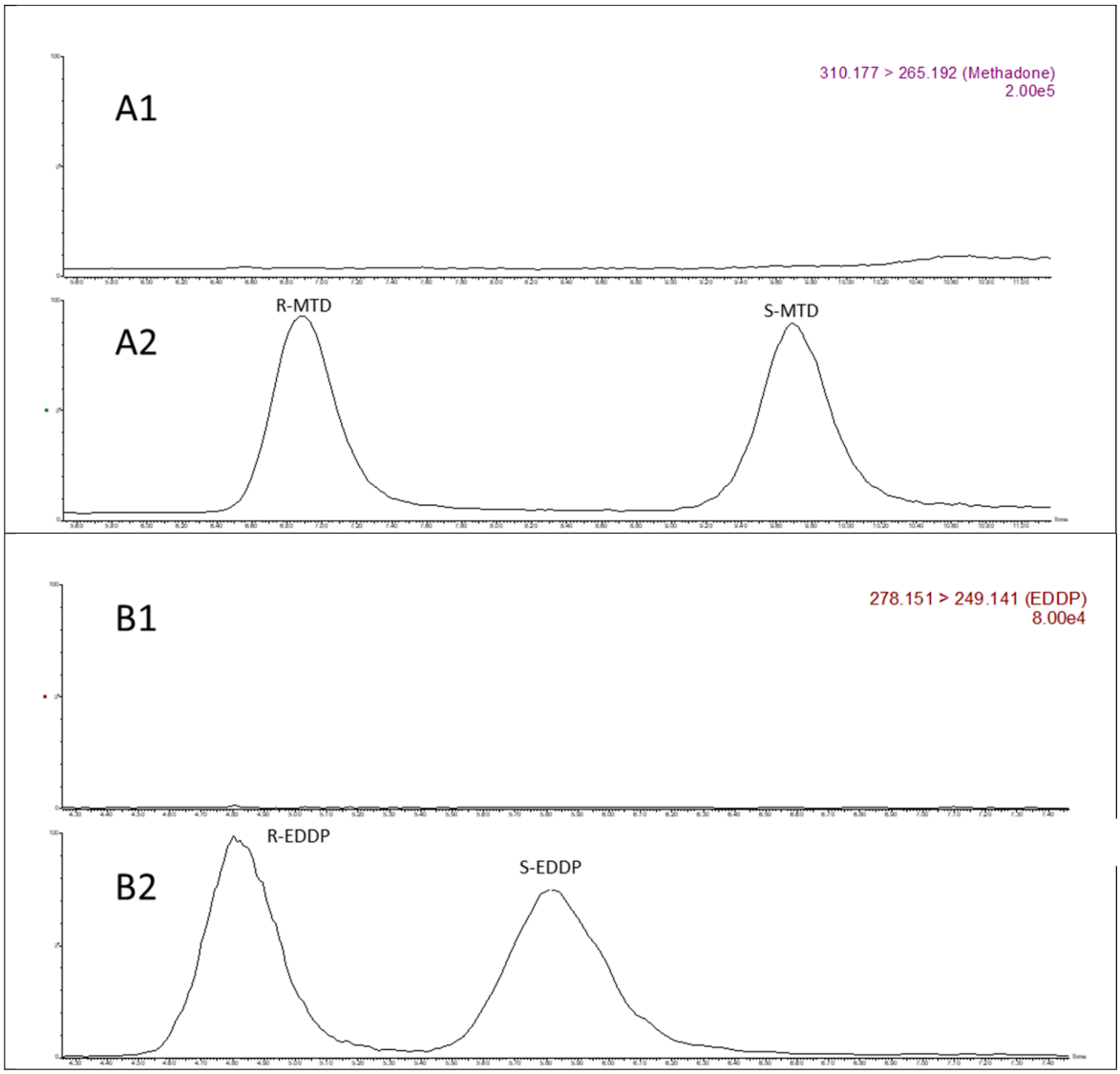

Fig. 3. R-/S-methadone and R-/S-EDDP enantiomeric separation in dried blood spot matrix obtained with UHPLC-MS/MS. Comparison between blank matrix and DBS fortified at the first point of the calibration curve (10 ng/mL for each enantiomer). (A1): Blank DBS matrix chromatogram compared to (A2) first calibration point at enantiomeric concentration of 10 ng/mL R- and S-methadone. (B1): Blank DBS matrix chromatogram compared to (B2) first calibration point at enantiomeric concentration of 10 ng/mL R- and S-EDDP.

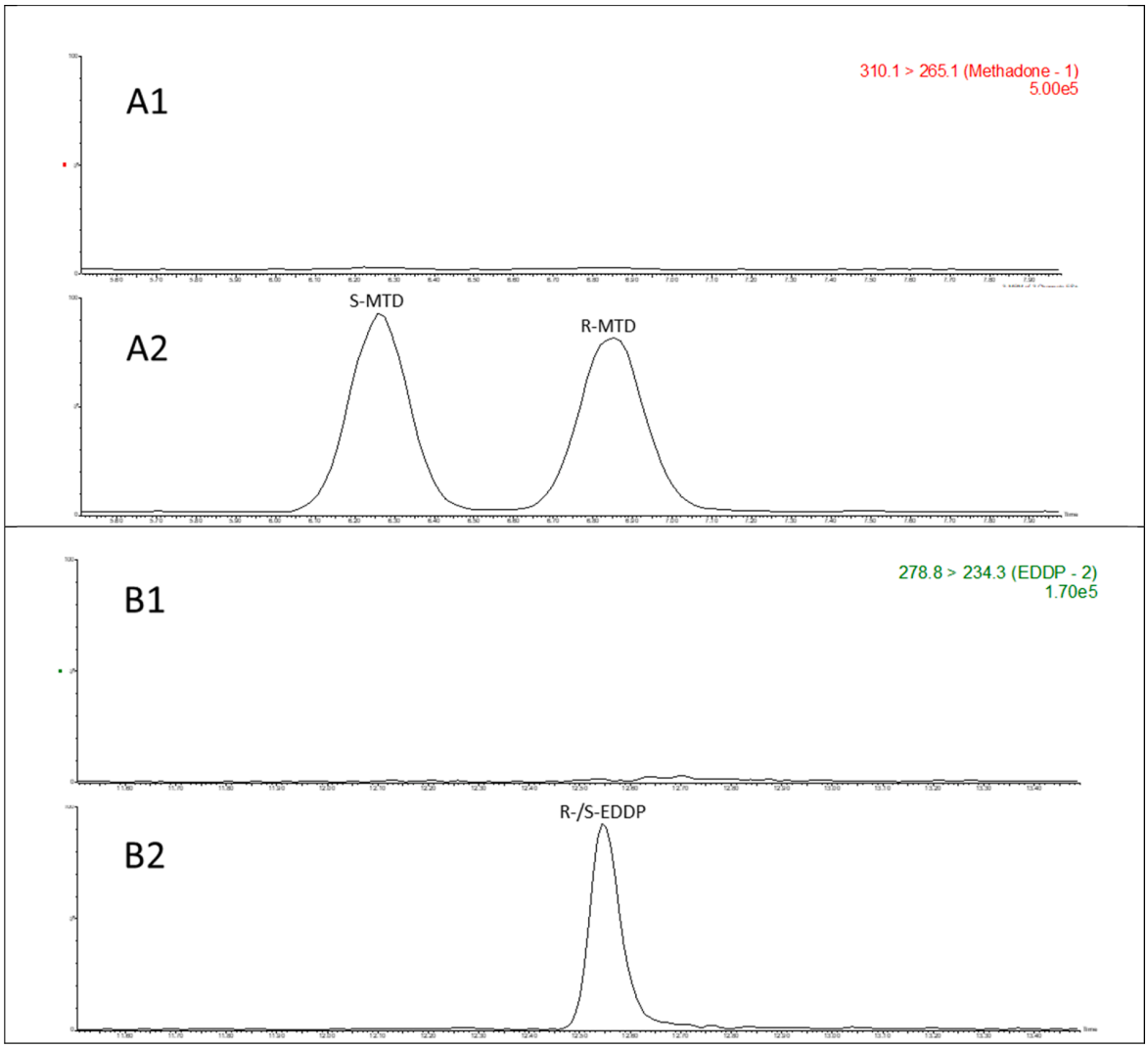

Fig. 4. R-/S-methadone enantiomeric separation and R-/S-EDDP chromatographic peak in dried blood spot matrix obtained with UHPSFC-MS/MS. Comparison between blank matrix and DBS fortified at the first point of the calibration curve (10 ng/mL for each enantiomer). (A1): Blank DBS matrix chromatogram compared to (A2) first calibration point at enantiomeric concentration of 10 ng/mL R- and S-methadone. (B1): Blank DBS matrix chromatogram compared to (B2) first calibration point at the concentration of 20 ng/mL R-/S-EDDP.

comprised between 85% and 91%. The matrix effects using SFC-MS/MS analysis were negligible and a slight ion suppression is observed using LC-MS/MS. No interferences were observed after the injection of six different human blood samples containing benzodiazepines, cocaine, amphetamines, opioids, antidepressants, neuroleptics and cannabinoids. Accuracy and precision for all samples used for stability assessment were in between ±15.0%. At least, methadone and EDDP on DBS were stable for 5 months when blood is stored as a dried blood spot at room temperature.

3.3. Human post-mortem samples: Results and comparison

Post-mortem dried matrix spots were analysed with both LC-MS/MS and SFC-MS/MS and results obtained for all samples were compared for the two bioanalytical methods and reported in Appendix 1. A significant spearman correlation is determined by comparing results obtained with the two methods for R-methadone, S-methadone (Appendix 1) and R-/S-EDDP (see Appendix 2) quantitation.

Median methadone concentration in cardiac blood was equal to 1217 ng/mL, while it was equal to 1038 ng/mL in peripheral blood (Table 5). R/S methadone ratio was found to be similar in all the four tested matrices and was in favour of the R- enantiomer form. Number (N°) of post-mortem samples for EDDP was not the same as for methadone, because samples results where EDDP was measured under the LOQ were not included. Total EDDP concentration in peripheral blood and cardiac blood was highly comparable (Table 5), with values of 285 ng/mL for the peripheral blood and 286 ng/mL for the cardiac blood, respectively. R- and S-EDDP ratio was similar in all four matrices and was in favour of the S-form.

R-methadone and S-methadone ratios between cardiac blood and peripheral blood showed a significant difference in favour of the cardiac blood (Table 6). R-methadone and S-methadone ratios between pericardial fluid and peripheral blood showed a significant difference in favour of the pericardial fluid (Table 6) for seven post-mortem cases where both fluids were available.

In Fig. 5, the correlation between R-methadone and R-EDDP measured in all post-mortem dried matrix spots analysed is shown. In all samples, R-methadone was more concentrated than R-EDDP, which was present in low quantity. Although several samples showed a slightly higher S-EDDP concentration compared to the S-methadone (Fig. 6), S-

Table 2
Validation parameters comparison for R-methadone quantitation methods using LC-MS/MS and SFC-MS/MS.

Validation parameter	Validation data LC	Validation data SFC
Calibration model	Wheighted linear curve 1/x, R-methadone-d9 as internal standard.	
	Eight point calibration curves with levels: 10, 25, 50, 100, 250, 500, 1000, 2500 ng/mL	
	Mean correlation coefficient (r2): **0.9992**	Mean correlation coefficient (r2): **0.9992**
Bias	LLOQ (10 ng/mL): **93.1%**	LLOQ (10 ng/mL): **97.5%**
	low (30 ng/mL): **98.0%**	low (30 ng/mL): **97.9%**
	medium (1000 ng/mL): **104.4%**	medium (1000 ng/mL): **102.0%**
	high (2000 ng/mL): **97.6%**	high (2000 ng/mL): **95.5%**
Precision	**Inter-day CV:**	**Inter-day CV:**
	LLOQ (10 ng/mL): **3.3%**	LLOQ (10 ng/mL): **10.5%**
	low (30 ng/mL): **4.4%**	low (30 ng/mL): **4.3%**
	medium (1000 ng/mL): **2.5%**	medium (1000 ng/mL): **5.1%**
	high (2000 ng/mL): **2.9%**	high (2000 ng/mL): **4.9%**
	Intra-day CV:	**Intra-day CV:**
	LLOQ (10 ng/mL): **3.3%**	LLOQ (10 ng/mL): **4.4%**
	low (30 ng/mL): **7.1%**	low (30 ng/mL): **8.0%**
	medium (1000 ng/mL): **3.1%**	medium (1000 ng/mL): **2.1%**
	high (2000 ng/mL): **5.8%**	high (2000 ng/mL): **4.0%**
Carry over	**No carryover** was observed after 3xULOQ (7500 ng/mL) after three injection repetition	
Interference studies	**No interfering signal** from matrix, internal standard, common drugs of abuse and prescription medications from 10 samples taken from 10 human sources.	
Recovery	**95–100%**	
Matrix effect	**98–100%**	**103–117%**
Limit of quantification (LOQ)	**10 ng/mL**	**10 ng/mL**
Limit of detection (LOD)	**2.5 ng/mL**	**0.5 ng/mL**
Selectivity, specificity	**No interferences**	
Standard Uncertainly (SD)	**14.2%**	**21.0%**

Table 3
Validation parameters comparison for S-methadone quantitation methods using UHPLC-MS/MS and UHPSFC-MS/MS.

Validation parameter	Validation data LC	Validation data SFC
Calibration model	Wheighted linear curve 1/x, S-methadone-d9 as internal standard.	
	Eight point calibration curves with levels: 10, 25, 50, 100, 250, 500, 1000, 2500 ng/mL	
	Mean correlation coefficient (r2): **0.9992**	Mean correlation coefficient (r2): **0.9991**
Bias	LLOQ (10 ng/mL): **94.2%**	LLOQ (10 ng/mL): **97.4%**
	low (30 ng/mL): **98.2%**	low (30 ng/mL): **97.9%**
	medium (1000 ng/mL): **104.8%**	medium (1000 ng/mL): **101.6%**
	high (2000 ng/mL): **97.0%**	high (2000 ng/mL): **94.5%**
Precision	**Inter-day CV:**	**Inter-day CV:**
	LLOQ (10 ng/mL): **6.3%**	LLOQ (10 ng/mL): **10.0%**
	low (30 ng/mL): **4.3%**	low (30 ng/mL): **4.6%**
	medium (1000 ng/mL): **2.8%**	medium (1000 ng/mL): **5.6%**
	high (2000 ng/mL): **3.4%**	high (2000 ng/mL): **5.2%**
	Intra-day CV:	**Intra-day CV:**
	LLOQ (10 ng/mL): **4.0%**	LLOQ (10 ng/mL): **4.4%**
	low (30 ng/mL): **6.8%**	low (30 ng/mL): **8.5%**
	medium (1000 ng/mL): **3.1%**	medium (1000 ng/mL): **3.2%**
	high (2000 ng/mL): **5.8%**	high (2000 ng/mL): **5.2%**
Carry over	**No carryover** was observed after 3xULOQ (7500 ng/mL) after three injection repetition	
Interference studies	**No interfering signal** from matrix, internal standard, common drugs of abuse and prescription medications from 10 samples taken from 10 human sources.	
Recovery	**95–100%**	
Matrix effect	**98–100%**	**103–117%**
Limit of quantification (LOQ)	**5 ng/mL**	**1 ng/mL**
Limit of detection (LOD)	**2.5 ng/mL**	**0.5 ng/mL**
Selectivity, specificity	**No interferences**	
Standard Uncertainly (SD)	**13.6%**	**20.0%**

methadone concentration measured in the majority of the post-mortem samples was higher compared to the S-EDDP.

4. Discussion

In the present study, we compared two fully validated bioanalytical methods involving two different chromatographic separation modes, namely LC-MS/MS and SFC-MS/MS. The same volume of 0.5 µL was injected in both LC and SFC systems and the two methods have the same analysis time of 14 min. We compared the method validation parameters and the bioanalytical quantitation results obtained with both methods for a wide range of post-mortem samples. Both methods were validated following the same guidelines and results are summarized in Tables 2, 3 And 4. LC-MS/MS method has a lower uncertainty for both methadone enantiomers, while SFC-MS/MS gives a lower LOD. Chromatogram showing the chiral separation of methadone in SFC-MS/MS (Fig. 4) suggests a lower signal to noise in blank matrix compared to that obtained in LC (Fig. 3), even if the signal intensity produced by the MS/MS device was lower compared to LC-MS/MS. In addition, the SFC-MS/MS method presented a better accuracy for both enantiomeric methadone enantiomers, whereas LC-MS/MS showed a greater precision (Tables 1 and 2). Methadone matrix effect was negligible in LC-MS/MS and a slight ion enhancement was observed in SFC-MS/MS. On the contrary, EDDP did not show any matrix effect in SFC-MS/MS compared to the

Table 4
Validation parameters comparison for R-/S-EDDP quantitation methods using UHPLC-MS/MS and UHPSFC-MS/MS.

Validation parameter	Validation data LC R-EDDP	Validation data LC S-EDDP	Validation data SFC R-/S-EDDP
Calibration model	Wheighted linear curve 1/x, R-/S-EDDP-d3 as internal standard.		
	Eight point calibration curves with levels: 10, 25, 50, 100L, 250, 500, 1000, 2500 ng/mL		Eight point calibration curves with levels: 20, 50, 100, 200, 500, 1000, 2000, 5000 ng/mL
	Mean correlation coefficient (r2): **0.9995**	Mean correlation coefficient (r2): **0.9995**	Mean correlation coefficient (r2): **0.9995**
Bias	LLOQ (10 ng/mL): **104.2%**	LLOQ (10 ng/mL): **104.4%**	LLOQ (20 ng/mL): **109.0%**
	low (30 ng/mL): **91.7%**	low (30 ng/mL): **92.0%**	low (60 ng/mL): **93.7%**
	medium (1000 ng/mL): **100.6%**	medium (1000 ng/mL): **100.4%**	medium (2000 ng/mL): **96.8%**
	high (2000 ng/mL): **92.8%**	high (2000 ng/mL): **92.9%**	high (4000 ng/mL): **91.8%**
Precision	**Inter-day CV:**	**Inter-day CV:**	**Inter-day CV:**
	LLOQ (10 ng/mL): **3.8%**	LLOQ (10 ng/mL): **4.5%**	LLOQ (20 ng/mL): **5.3%**
	low (300 ng/mL): **3.9%**	low (300 ng/mL): **3.9%**	low (60 ng/mL): **3.2%**
	medium (1000 ng/mL): **3.8%**	medium (1000 ng/mL): **3.7%**	medium (2000 ng/mL): **4.1%**
	high (2000 ng/mL): **3.5%**	high (2000 ng/mL): **3.6%**	high (4000 ng/mL): **3.7%**
	Intra-day CV:	**Intra-day CV:**	**Intra-day CV:**
	LLOQ (10 ng/mL): **4.2%**	LLOQ (10 ng/mL): **4.0%**	LLOQ (20 ng/mL): **3.9%**
	low (300 ng/mL): **3.4%**	low (300 ng/mL): **3.7%**	low (60 ng/mL): **3.0%**
	medium (1000 ng/mL): **2.9%**	medium (1000 ng/mL): **2.6%**	medium (2000 ng/mL): **4.1%**
	high (2000 ng/mL): **3.4%**	high (2000 ng/mL): **3.5%**	high (4000 ng/mL): **3.0%**
Carry over	**No carryover** was observed after 3xULOQ (7500 ng/mL) after three injection repetition		
Interference studies	**No interfering signal** from matrix, internal standard, common drugs of abuse and prescription medications from 10 samples taken from 10 human sources.		
Recovery	**85–91%**		
Matrix effect	**91–93%**		**97–103%**
Limit of quantification (LOQ)	**10 ng/mL**		**20 ng/mL**
Limit of detection (LOD)	**1.5 ng/mL**		**0.5 ng/mL**
Selectivity, specificity	**No interferences**		
Standard Uncertainly (SD)	**16.6%**	**16.0%**	**18.0%**

slight ion suppression observed in LC-MS/MS. Though both methods separated the chiral methadone completely, the enantiomeric methadone separation in LC-MS/MS (Table 1) showed a greater resolution compared to that obtained in SFC-MS/MS.

From our knowledge, although a very limited number of publications [22–25] compared validation parameters between LC and SFC for bioanalytical methods on achiral and chiral compounds, results from real human samples were never compared until now. The present study provides the comparison between results obtained from 93 samples containing methadone. In Appendix 1, a very high correlation coefficient was obtained when comparing results obtained with the two methods, $r_s = 0.9977$ for R-methadone and $r_s = 0.9978$ for S-methadone. Interestingly, results are consistent for the three tested matrices and from low to high concentration levels, confirming the excellent linearity and accuracy of both methods. As highlighted in this work, although the enantiomeric EDDP separation was not achieved in SFC-MS/MS, the supercritical chromatography could be considered as an alternative to LC-MS/MS for the quantitative analysis of methadone enantiomers in biological fluids. Underlying the results obtained in our study, SFC-MS/MS should also be considered in other forensic routine applications, as a valid instrumentation for biological samples analysis.

Until now, only four studies were published describing the enantiomeric methadone separation in post-mortem samples, such as blood and tissues [8,9,31,32]. These studies used the same LC-MS/MS conditions for the enantiomeric separation and detection. This study provides the first R- and S-methadone quantitation using an SFC-MS/MS approach with dried matrix spot. Median R-/S-methadone ratio of 1.60 (0.79 – 4.23) and R-/S-EDDP of 0.84 (0.45 – 1.32) measured in peripheral blood are in agreement with previously published data [8,31]. Pharmacokinetics studies demonstrated the stereoselective CYP450 metabolism offered a longer half-life and a larger volume of distribution for R-methadone compared to the S-methadone [33–35]. Considering the higher cardiotoxicity of the S-methadone compared to the R-methadone [5], a novel compound could be developed to increase S-methadone metabolism by improving the S-stereoselective CYP2B6 enzyme activity [2].

In Table 6, we showed that ratio between pericardial fluid and peripheral blood was in favour of the former, with a significant correlation. Since scientists [36–38] revealed that pericardial fluid is a quite isolated compartment for different substances, similar to vitreous humour, this property could be verified for R-/S-methadone, by comparing pericardial fluid results with other isolated compartments, such as

Table 6
Ratio between cardiac blood and peripheral blood (CB/PB) with significant spearman correlation coefficient (R-methadone: $r_s = 0.7962$; $p < 0.0001$), (S-methadone: $r_s = 0.7655$; $p < 0.0001$). Ratio between pericardial fluid and peripheral blood (PF/PB) with significant spearman correlation (R-methadone: $r_s = 0.8929$; $p = 0.006$), (S-methadone: $r_s = 0.7143$; $p = 0.04$).

	N 27	CB/PB	N 7	PF/PB
R-MTD		**1.31**		**1.49**
S-MTD		**1.36**		**1.91**
Total MTD		**1.33**		**1.64**

Table 5
Median and ranges for total methadone and EDDP as well as R/S ratio found in the four examined matrices.

	N	Total MTD [ng/mL]	R/S-MTD ratio	N	Total EDDP [ng/mL]	R/S-EDDP ratio
Femoral blood	32	**1038** (32–5000)	**1.60** (0.79–4.23)	27	**285** (34–1421)	**0.84** (0.45–1.32)
Cardiac blood	29	**1217** (38–6900)	**1.63** (1.03–4.36)	28	**284** (32–1493)	**0.76** (0.56–1.07)
Pericardiac Fluid	9	**851** (89–3420)	**1.79** (0.81–4.22)	7	**257** (36–679)	**0.68** (0.52–1.02)
Vitreus	23	**145** (17–671)	**1.88** (1.11–4.67)	4	**65** (35–122)	**0.77** (0.68–0.84)

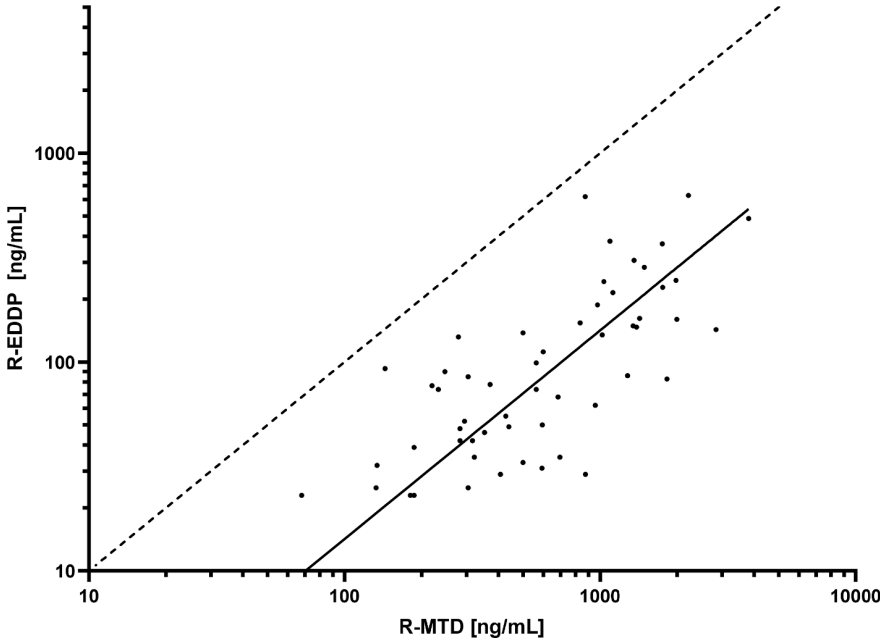

Fig. 5. Correlation between R-methadone and R-EDDP in all post-mortem analysis. Spearman correlation coefficient (r_s) was found to be significant with a $p < 0.0001$ and $r_s = 0.7308$.

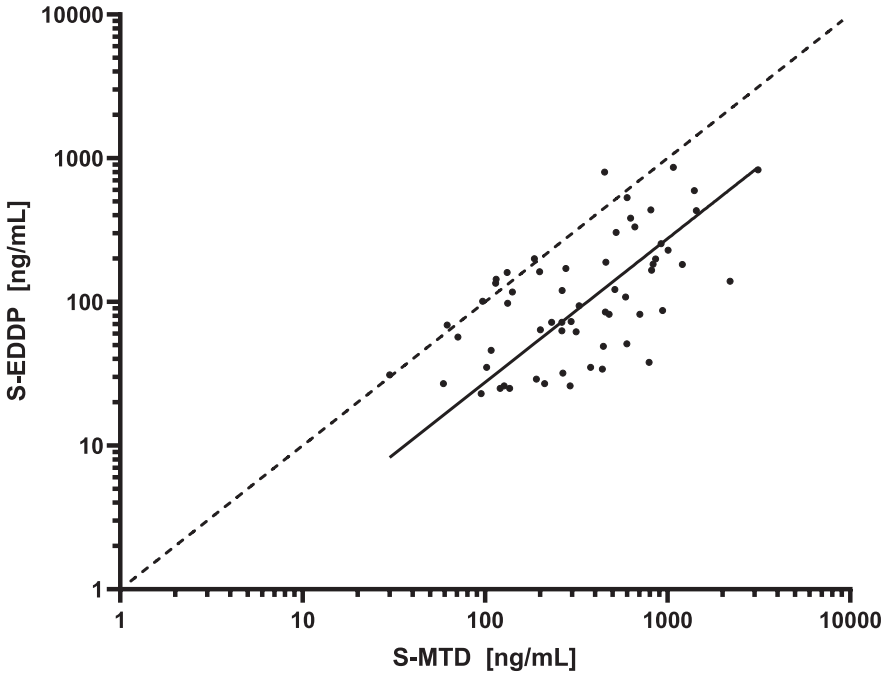

Fig. 6. Correlation between S-methadone and S-EDDP in all post-mortem analysis. Spearman correlation coefficient (r_s) was found to be significant with a $p < 0.0001$ and $r_s = 0.6196$.

cerebrospinal fluid [39], or by following the methadone distribution in pericardial fluid over time in post-mortem cases [38]. In vitreous humour, methadone and EDDP concentrations were lower than in the others analysed matrices (Table 5), probably due to the high hydrophobic property of both substances. Median of methadone concentration in vitreous humor of 145 ng/mL (N = 23) was in agreement with those obtained by Fernandez et al. [40] at 110 ng/mL (N = 5), while EDDP concentration totally disagree from the two studies. Median EDDP concentration obtained by Fernandez et al. [40] was 680 ng/mL (N = 5), whereas in our study it was only 65 ng/mL (N = 4) and, furthermore, results are probably lower because 19 samples presented EDDP concentration lower than the LOQ and were not included in the results. Our results for EDDP concentration in vitreous humor are in agreement with the results obtained in routine analysis on post mortem cases and with the relationship between methadone and EDDP in other human matrices [8,9,31,32].

Ratio between R-methadone and its main metabolite R-EDDP are shown to be in favour of the first one (Fig. 5). Although less significant,

ratio between S-methadone and S-EDDP showed the same behaviour (Fig. 6). R-methadone has a greater half-life time compared to S-methadone and probably, both have a longer half-life time compared to their main enantiomers metabolites, resulting in a higher concentration in almost all analysed matrices [2]. Besides polymorphic genetics in CYP450 enzymes, scientist have shown correlations between EDDP elimination and urine pH [41]. They have highlighted that as the urinary pH increases, the proportion of excreted EDDP increases.

5. Conclusion

This study described the development of LC-MS/MS and SFC-MS/MS enantiomeric methadone quantitation methods and compared their quantitative performance in real post-mortem dried matrix spots from blood, vitreous humor and pericardial fluid. Methods validation comparison and correlations graphs between results obtained with both methods confirms that SFC-MS/MS could be taken in consideration as an alternative to the widely used LC-MS/MS for bioanalytical methods development and validation. Both methods presented in this study can be easily and quickly used in toxicological routine analysis for the methadone quantitation in human fluids matrices, but the polysaccharide coated column IH-3 used in SFC does not allow the enantiomeric EDDP separation.

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CRediT authorship contribution statement

F. Mueller: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Validation, Visualization, Writing - original draft, Writing - review & editing. **G.L. Losacco:** Data curation, Investigation, Software, Visualization, Writing - review & editing. **R. Nicoli:** Investigation, Resources, Supervision, Visualization, Writing - review & editing. **D. Guillarme:** Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing - review & editing. **A. Thomas:** Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing - review & editing. **E. Grata:** Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2021.122755.

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IV.2 Using 1.5 mm internal diameter columns for optimal compatibility with current LC systems

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Using 1.5 mm internal diameter columns for optimal compatibility with current liquid chromatographic systems

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ABSTRACT

This article describes the use of a new prototype column hardware made with 1.5 mm internal diameter (i.d.) and demonstrates some benefits over the 1.0 mm i.d. micro-bore column. The performance of 2.1, 1.5 and 1.0 mm i.d. columns were systematically compared. With the 1.5 mm i.d. column, the loss of apparent column efficiency can be significantly reduced compared to 1.0 mm i.d. columns in both isocratic and gradient elution modes. In the end, the 1.5 mm i.d. column is almost comparable to 2.1 mm i.d. column from a peak broadening point of view. The advantages of the 1.5 mm i.d. hardware vs 2.1 mm i.d. narrow-bore columns are the lower sample and solvent consumption, and reduced frictional heating effects due to decreased operating flow rates.

1. Introduction

Analytical scale liquid chromatographic (LC) columns are commercially available in 4.6, 3.9, 3.0, 2.1, 2.0 and 1.0 mm internal diameters (i.d.). Terms such as standard-bore (4.6, 3.9 and 3.0 mm i.d.), narrow-bore (2.1 and 2.0 mm i.d.) and micro-bore (1.0 mm i.d.), are often used to describe and distinguish the different column formats and the required operating flow rate [1,2,3]. Columns based on 3.0 – 4.6 mm have historically dominated the field of chromatography however, there has been a significant increase in using 2.1 mm i.d. columns, largely due to the adoption of ultra-high pressure liquid chromatographic (UHPLC) technology and UHPLC–MS systems [3]. Conversely, the use of 1.0 mm i.d. column format is still not widely adopted.

As possible advantages, smaller column diameters result in lower solvent consumption, thus reducing the cost of analysis and offering a greener solution [4]. On small diameter columns, the optimal flow rate is lower, therefore frictional heat effects become less important, and give rise to more efficient desolvation for LC–MS analyses, resulting in higher sensitivity. Finally, the sample consumption is also reduced with smaller columns, since injected volume has to be scaled in direct proportion with the column volume, to maintain the same sensitivity.

On the other hand, the disadvantages of micro-bore columns may lie in limited loading capacity and decreased apparent efficiency due to extra-column band broadening. The limited loading capacity is often not very critical, but the loss of apparent column efficiency can be serious. Lestremau and co-workers compared the apparent efficiency of 1.0 × 100 mm and 2.1 × 100 mm columns packed with the same material - using a modern UHPLC system – and only about 67% of the efficiency was obtained on the 1.0 mm i.d. column compared to the 2.1 mm i.d. one, in isocratic mode [4]. The apparent efficiency could be improved by increasing the column length and therefore the ratio of column volume to system volume. In gradient mode, the contribution of the extra-column bandspreading was significantly reduced and a peak capacity of about 80% of their equivalent 2.1 mm i.d. columns can be obtained on the 1.0 mm i.d. column [4]. Wu and Bradley reported about 60% drop in plate numbers (N) when comparing 2.1 × 50 mm and 1.0 × 50 mm columns packed with 1.8 µm particles and operating them under isocratic conditions (N = 9010 plates vs. 3580) [5]. They concluded that the efficiency loss due to extra-column band broadening increases as the column diameter and column length decrease. This effect was even more pronounced for early eluting components. Another study also reported that the extra-column

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dispersion of a given LC system can dramatically decrease the apparent performance of highly efficient narrow-bore columns [6]. To properly operate 2.1 × 50 mm columns, an LC system possessing system dispersion as low as $\sigma^2 \leq 10$ µL^2 is required to maintain at least 55% of intrinsic column efficiency. When coupling micro-bore columns to MS, the tubing used to interface the UHPLC system to the MS device is particularly critical in both isocratic and gradient modes, because this tubing is located after the column outlet, where the band compression effects that compensate for band broadening do not occur [7]. Standard commercial UHPLC–MS instruments (unmodified) exhibit σ^2 values ranging from 20 to more than 100 µL^2. However, by minimizing the volume of the interfacing tube, the extra-column variance can be reduced to $\sigma^2 \leq 20$ µL^2 for any type of MS detector (please note that routinely, very long interfacing tubes are used in practice, such as 50 – 100 cm). With an optimized UHPLC–MS configuration, the loss in efficiency with a 2.1 mm I.D. column was negligible at retention factors (k) higher than 7, while the 1 mm I.D. column was hardly compatible with current instrumentation, even at $k > 20$. The impact of the extra-column band broadening on the chromatographic peaks in gradient mode was subtle, though still unacceptable with micro-bore columns [7].

The lower efficiency of 1.0 mm i.d. columns is not exclusively due to extra-column dispersion but can also be caused by poorly packed beds, frit dispersion and axial bed heterogeneity. Gritti and Wahab reported that the packed bed near the wall of the column is denser than that of the bulk packing, which results in differences in both solute and mobile phase velocity through the column [8]. For a 1.0 mm i.d. column, the wall region volume (denser) to bulk region volume (less dense) ratio is close to 1 which is the worst case. For larger-diameter columns, the wall region volume becomes less significant and - for smaller column internal diameter - the bulk region becomes less significant. Thus, the 1.0 mm i.d. is often considered to be the worst-case from bed heterogeneity point of view.

Despite the expectedly high efficiency loss with 1.0 mm i.d. columns, these columns can be used by utilizing carefully optimized systems, optimal column hardware design, very low injected volume and by benefiting from band focusing effects using weak injection solvent [9,10]. Schoors and co-workers reported the successful usage of 1.0 mm i.d. columns for the analysis of monoamine neurotransmitters [11]. They demonstrated a significant increase in sensitivity using 1.0 mm i.d. column as compared to a 2.1 mm i.d. column. In addition, weak solvent injection helped focusing the sample at the column inlet.

It seems today that despite the sensitivity increase and reduced solvent consumption, the adoption of micro-bore columns is still limited. The efforts needed to compensate for system band broadening seem to be a strong barrier for most users. The aim of this study was to evaluate a compromise between 1.0 and 2.1 mm columns. Thus, a prototype 1.5 mm i.d. column was prepared and systematically compared to commercial 2.1 and 1.0 mm i.d. columns packed with a highly efficient column packing material (2.7 µm superficially porous 90 Å C18). Column efficiency observed in both isocratic and gradient modes were compared using a very low dispersion (Acquity I-Class) and a standard UHPLC (Acquity H–Class) systems. In addition, the sensitivity of MS detection was also studied.

2. Experimental

2.1. Chemicals and samples

Acetonitrile (AcN), methanol (MeOH), ethanol (EtOH), water and formic acid were purchased from Fisher Scientific (Reinach, Switzerland). Uracil, methylparaben, ethylparaben, propylparaben, butyl–paraben, cannabidivarine (CBDV), cannabigerolic acid (CBGA), tetrahydrocannabivarin (THCV), cannabichromene (CBC), delta9-tetrahydrocannabinolic acid (THCA-A) and human serum albumin (HSA), were purchased from Sigma–Aldrich. Cannabidiolic acid (CBDA), cannabigerol (CBG), cannabidiol (CBD), cannabinol (CBN), (-)-delta9-THC (d9-THC) and (-)-delta8-THC (d8-THC) were purchased from Lipomed AG (Arlesheim, Switzerland). Terbutaline, fenfluramine, norfentanyl, atenolol, benzoylecgonine, probenecid, hydrochlorothiazide, etacrynic acid, furosemide, chlorthalidone, bumetanide and bendroflumethiazide solution at 1 mg/mL in MeOH were kindly provided by the Swiss Laboratory for Doping Analyses (Epalinges, Switzerland).

2.2. Chromatographic system

For UHPLC-UV measurements, two UHPLC systems were used. One was a very low-dispersion system, namely a Waters Acquity UPLC I-Class (Waters, Milford, MA, USA) equipped with a binary solvent delivery pump, an autosampler and UV detector. The system included a flow through needle (FTN) injection system with 15 µL needle and a 0.5 µL UV flow-cell. The extra-column volume of the system was measured as $V_{ec} = 7.5$ µL, while the gradient delay volume was $V_d = 98$ µL. The other system was a Waters Acquity UPLC H–Class equipped with a quaternary solvent delivery pump, an autosampler and UV detector. The system included a FTN injection system with 15 µL needle and a 0.5 µL UV flow-cell. The extra-column volume of the system was measured as $V_{ec} = 11.5$ µL, while the gradient delay volume was $V_d = 370$ µL.

For UHPLC-MS/MS measurements, a third Waters Acquity UPLC I-Class, composed of a binary solvent delivery pump and an autosampler (loop offline), was hyphenated to a Waters TQD Triple Quadrupole mass spectrometer, fitted with a Z-spray ESI source. A capillary voltage of ±1.5 kV, source temperature of 150 °C, desolvation temperature at 450 °C, desolvation and cone gas set at 750 L/h and 0 L/h were applied to all analyses. Nitrogen (N_2) was used as both desolvation and cone gas, while argon (Ar) was employed as the collision gas. Multiple reaction monitoring (MRM) mode was used during UHPLC-MS analyses. The UHPLC system was connected to the MS via 65 µm x 50 cm PEEK tube.

Data acquisition and instrument control for UHPLC-UV measurements were performed by Empower Pro 3 software (Waters, Milford, MA, USA), while for UHPLC-MS analyses, MassLynx v4.1 (Waters, Milford, MA, USA) was used. Data was treated in Excel (Microsoft) for UHPLC-UV analyses, while TargetLynx v4.1 (Waters, Milford, MA, USA) was used for UHPLC-MS measurements.

2.3. Columns

A new prototype 1.5 × 100 mm column packed with 2.7 µm superficially porous 90 Å C18 particles and commercial 1.0 × 100 mm and 2.1 × 100 mm columns packed with the same material were provided by Advanced Materials Technology (Wilmington, DE, USA).

2.4. Sample and mobile phase preparation

A mix solution containing uracil, methylparaben, ethylparaben, propylparaben and butylparaben was prepared in 10:90 v/v acetonitrile:water at 50 µg/mL. Uracil and parabens were eluted in the mobile phase, namely 35:65 v/v acetonitrile:water.

A mix solution containing eleven cannabinoids (i.e. CBDV, CBGA, THCV, CBC, THCA-A, CBDA, CBG, CBD, CBN, d9-THC and d8-THC) was prepared from individual stock solutions diluted in solvent having the same composition as mobile phase "A" at 45 µg/mL. The individual stock solutions were prepared in either methanol, acetonitrile or ethanol depending on their solubility. Cannabinoids

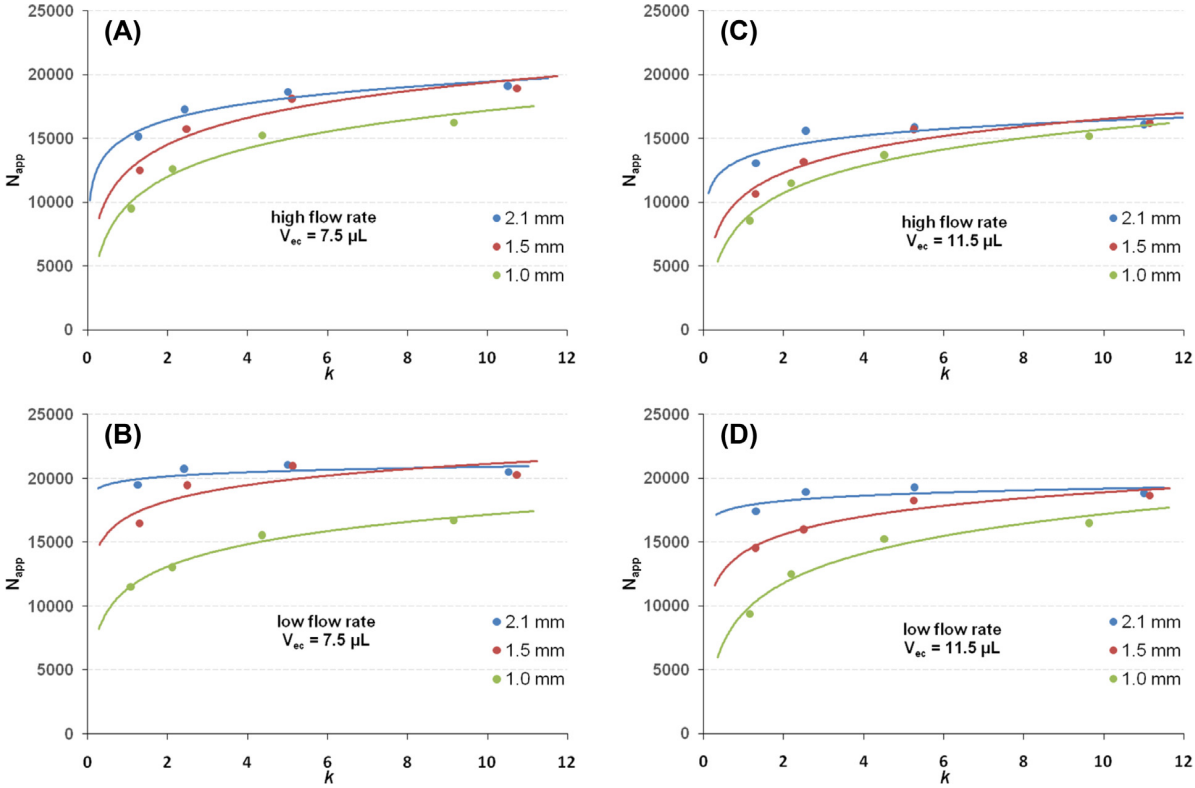

Fig. 1. Apparent plate numbers (N_{app}) as function of solute retention factor (k) observed on Acquity I-Class system at high (A) and low flow rates (B) and on H-class system at high (C) and low (D) flow rates with 1.0, 1.5 and 2.1 mm i.d columns. Sample: methyl-, ethyl-, propyl- and butyl–paraben.

were separated in gradient mode. Mobile phase "A" was 0.1% formic acid in water, while mobile phase "B" was 0.1% formic acid in acetonitrile. A linear gradient of 60 – 95% B was applied at two different gradient steepness (corresponding to gradient times of t_{G1} = 10 min and t_{G2} = 20 min), since peak width depends on gradient steepness.

For UHPLC-MS analyses, two mixtures of doping agents were used. Both mixtures were prepared in 5:95 v/v methanol:water at a final concentration of 200 pg/mL. Analyses were performed in gradient mode. Mobile phase "A" was 0.1% formic acid in water, while mobile phase "B" was 0.1% formic acid in acetonitrile. A linear gradient from 5% to 95% of mobile phase "B" was applied at two different gradient steepness (t_{G1} = 5 min for all analyses at high-flow rates on each column, t_{G2} = 3.3 min for all analyses at low-flow rates).

2.5. Comparison of efficiency

The linear mobile phase velocity (u_0) and the total column porosity (ε_T) were determined from the following equation:

$$u_0 = \frac{L}{t_0} = \frac{4F}{\varepsilon_T d_c^2 \pi} \quad (1)$$

where L is the nominal column length, t_0 is the column dead time (corrected for system residence time), F is the mobile phase flow rate, and d_c the nominal column diameter. The column dead time was measured by injecting non-retained compound (uracil).

When comparing column efficiency, two mobile phase velocities were set (low and high level). Linear velocities u_0 = 15 and 25 cm/min were considered, corresponding to F ~ 0.3 and 0.5 mL/min on the 2.1 mm i.d., F ~ 0.15 and 0.26 mL/min on the 1.5 mm i.d. and F ~ 0.07 and 0.11 mL/min on the 1.0 mm i.d. column. Mobile phase temperature was set to 30 °C. In both isocratic and gradient modes, three injection volumes were tested (V_{inj} = 0.1, 0.5 and 1µL) since injected volume might impact both system and column band-broadening, especially for small volume columns.

In isocratic mode, the apparent plate numbers (N_{app}, not corrected for system dispersion) obtained with the paraben mixture were compared. Plots of N_{app} versus retention factor (k) were plotted and logarithmic trends were fitted. The retention factors of the parabens were comprised between k ~ 1 and 11, which is representative of common practice. Since in isocratic mode, both the pre- and post column dispersions impact the total band broadening, two UHPLC systems were used to see the differences of apparent efficiency.

In gradient mode, the peak widths of cannabinoids measured at half height ($w_{1/2}$) were compared and plotted as a function of apparent retention factor (k_{app}, based on observed retention time). The gradient measurements were performed only on the low-dispersion system. Please note that in gradient mode, it is mostly the post column volume that impacts the overall peak broadening, since the pre-column dispersion is compensated by the gradient band focusing effect. The post column volume of the low dispersion (Acquity I-Class) and a standard UHPLC (Acquity H–Class) systems were the same (0.5 µL UV cell and post column connector tubing of 65 µm x 30 cm), thus why only one system was tested here.

2.6. Comparison of sensitivity

Peak integration and signal-to-noise (S/N) measurements were performed via the TargetLynx tool available in MassLynx v4.1. Smoothing process was applied to all chromatograms prior to all

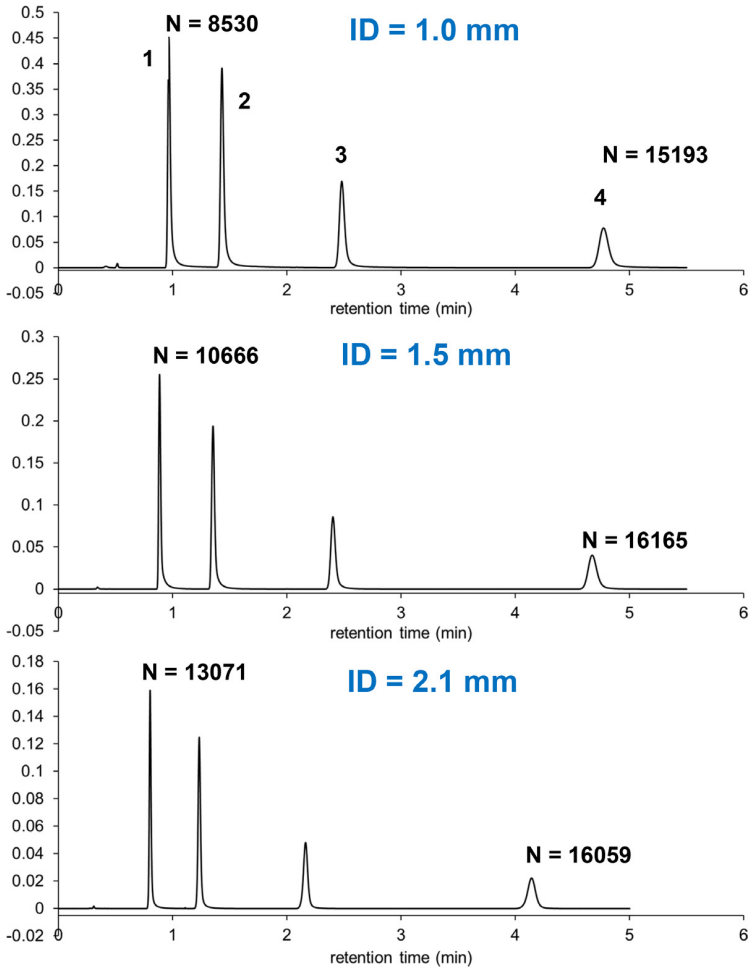

Fig. 2. Comparative chromatograms, obtained with 1.0 × 100 mm, 1.5 × 100 mm and 2.1 × 100 mm columns - packed with superficially porous C18 material - in isocratic mode (standard UHPLC, Acquity H–Class). Mobile phase 35:65 v/v acetonitrile:water, mobile phase velocity u_0 = 25 cm/min. Sample: methyl-paraben (1), ethyl-paraben (2), propyl-paraben (3) and butyl–paraben (4).

calculations, using a Savitzky-Golay method with a smoothing iteration value of 3 and a smoothing width of 2. The "Peak-to-Peak" method was applied for signal-to-noise calculation, measuring peak signal level from the baseline and by fixing a constant noise signal window of 1.0 min for all chromatograms.

3. Results and discussion

In the first instance, the column volumes and porosities were determined. Column volumes, V_0, were equal to 173, 100 and 49 µL, while porosities ε_T were equal to 0.50, 0.57 and 0.63 for the 2.1, 1.5 and 1.0 mm i.d. columns, respectively.

Regarding V_0, it is important to keep in mind the general 10% rule of thumb for extra-column band broadening and associated efficiency loss [12]: the extra-column volume should not be more than 10% of the column's packed bed volume (to limit the contribution of system dispersion). The extra-column volume of our very low dispersion UHPLC system is V_{ec} = 7.5 µL, which corresponds to 4, 7 and 15% of the 2.1, 1.5 and 1.0 mm i.d. columns volume (100 mm length), respectively. On the other hand, the system volume of our standard UHPLC system is V_{ec} = 11.5 µL, which corresponds to 7, 11 and 23% of the 2.1, 1.5 and 1.0 mm i.d. columns volume (100 mm length), respectively. These values suggest that the 1.5 × 100 mm column can be operated on a very low dispersion system without significant efficiency loss, while on a standard UHPLC, lower apparent efficiency is expected, but to a much lesser extent than on a 1.0 × 100 mm column. To operate a 2.1 × 100 mm i.d. column (packed with superficially porous particles) without significant efficiency loss, a system volume of $V_{ec} \leq$ 17 µL is required. For a 1.5 and 1.0 × 100 mm column, $V_{ec} \leq$ 10 µL, and $V_{ec} \leq$ 5 µL are recommended, respectively. This latter criterion ($V_{ec} \leq$ 5 µL) is problematic, since commercial UHPLC systems all possess $V_{ec} >$ 6–7 µL (typically between 6 and 20 µL) [6,7,12].

It is also worth mentioning that the observed column porosity increases when decreasing the column diameter. This observation is logical and can probably be explained by the following reasons: The apparent porosity of small columns increases when decreasing column diameter or length, due to the higher ratio of extra-bed volume (V_{eb}) to packed bed volume and due to some differences in packing quality (density) too [13,14]. The extra-bed volume of a column hardware was recently described as the total volume of column hardware flow distributor, flow collector, frits, and inlet/outlet connections [13,14]. To have an idea about its value, a recent study reported extra-bed volume of V_{eb} ~ 4 µL for a 2.1 mm i.d. column hardware [14]. Therefore, for very low volume columns (such as short columns of 1.0, 1.5 and 2.1 mm i.d.), not only the extra-column system volume, but also the extra-bed column volume need to be considered as possible source of efficiency loss.

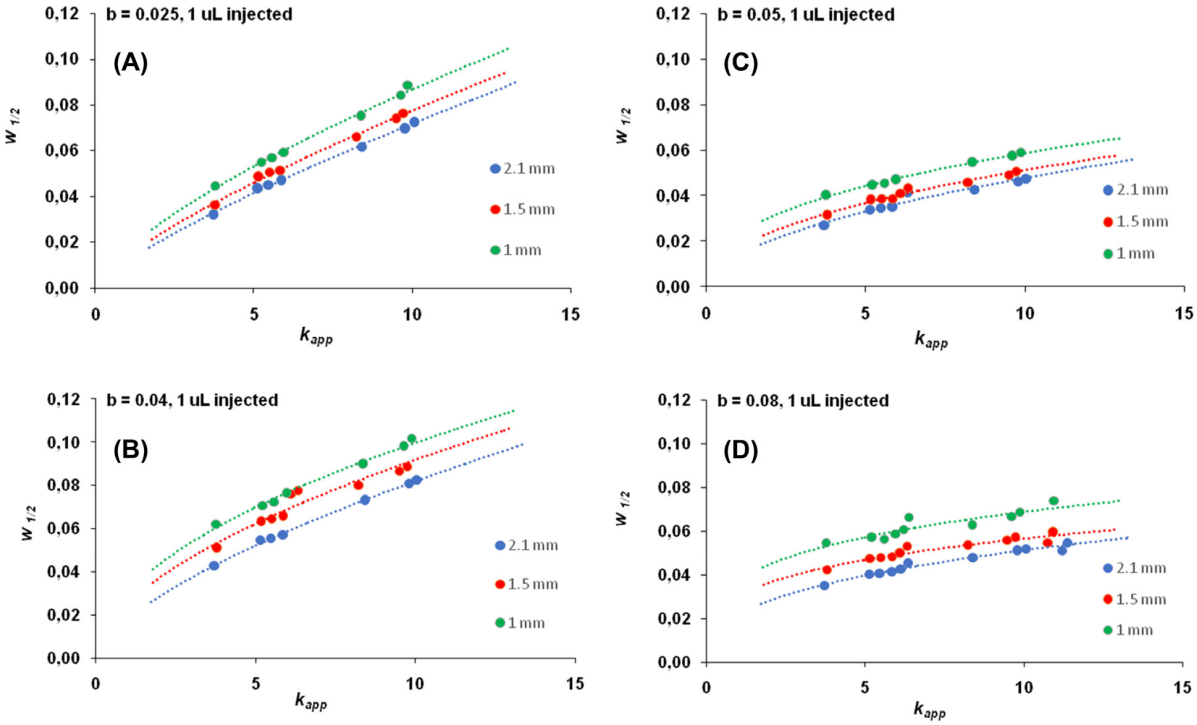

Fig. 3. Peak widths ($w_{1/2}$) as function of solute apparent retention factor (k_{app}) observed at b = 0.025 (A), 0.04 (B), 0.05 (C) and 0.08 (D) gradient steepnesses with 1.0, 1.5 and 2.1 mm i.d columns. Sample: cannabinoinds standard mixture. The widths of only the well-resolved peaks are considered. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.1. Apparent efficiency in isocratic mode

Fig. 1 shows the measured plate numbers (N_{app}) as a function of solute retention factor (k). Figs. 1A and B correspond to the very low dispersion system. At high flow rate, N_{app} = 15,000 – 19,000 plates were obtained on the 2.1 mm i.d. column. The 1.5 mm i.d. column resulted in N_{app} = 12,500 - 19,000 and the 1.0 mm i.d. column provided N_{app} = 9500 – 16,000. In average, the 1.5 and 1.0 mm i.d. columns performed ~93% and ~76% efficiency compared to the 2.1 mm i.d. column, respectively. At low flow rate, N_{app} = 19,500 – 21,000 (2.1 mm i.d.), N_{app} = 16,500 – 21,000 (1.5 mm i.d.) and N_{app} = 11,500 – 16,700 (1.0 mm i.d.) were observed. In average, we observed ~94% and 69% efficiency with the 1.5 and 1.0 mm i.d. columns, respectively, compared to the 2.1 mm i.d. column.

It can also be seen that the less retained peaks are more affected by system dispersion. This is obviously due to the fact that column peak variance (σ^2_{col}) - and therefore peak width - depends on solute retention:

$$\sigma^2_{col} = \frac{V_0^2}{N_{col}}(1+k)^2 \quad (2)$$

where N_{col} is the column intrinsic efficiency (plate number unaffected by system dispersion). Therefore, at low k, the ratio of system dispersion to column dispersion increases due to the decrease of column dispersion. When comparing the efficiency corresponding to poorly (k ~ 1) and highly retained (k ~ 10) compounds, the efficiency obtained for the poorly retained compound drops by about 20, 35 and 40% on the 2.1, 1.5 and 1.0 mm i.d. columns, respectively, at low flow rate. Similarly, at high flow rate, this efficiency loss corresponds to 7% (2.1 mm i.d.), 17% (1.5 mm i.d.) and 31% (1.0 mm i.d.).

Figs. 1C and D show the apparent efficiency obtained on a standard UHPLC system. At high flow rate, N_{app} = 13,000 – 16,000 plates were observed with the 2.1 mm i.d. column. The 1.5 mm i.d. column provided in N_{app} = 10,500 – 16,000, while the 1.0 mm i.d. column performed N_{app} = 8500 – 15,000. In average, the 1.5 mm i.d. column performed ~92% while the 1.0 mm i.d. column performed ~88% efficiency compared to the 2.1 mm i.d. column. At low flow rate, N_{app} = 17,500 – 19,000 (2.1 mm i.d.), N_{app} = 14,200 – 18,500 (1.5 mm i.d.) and N_{app} = 8200 – 16,000 (1.0 mm i.d.) were observed. In average, we obtained ~90% and 80% efficiency with the 1.5 mm and 1.0 mm i.d. columns, respectively, compared to the 2.1 mm i.d. column. Between poorly and highly retained solutes, at high flow rates, we saw 19, 34 and 43% difference in plate numbers on the 2.1, 1.5 and 1.0 mm i.d. columns, respectively. At low flow rate, we observed 8% (2.1 mm), 23% (1.5 mm) and 49% (1.0 mm) differences in plate numbers between k ~ 1 and 10.

As an example, Fig. 2 shows corresponding chromatograms obtained with the standard UHPLC system operating at high flow rate. It is worth mentioning that not only plate numbers, but peak symmetry is also affected by the column diameter. The poorly retained compounds elute in more asymmetrical peaks on smaller bore columns.

3.2. Apparent efficiency in gradient mode

In gradient elution mode, the apparent efficiency is expectedly less affected by system dispersion, since most of the dispersion occurring in the pre-column volumes are compensated by band focusing taking place at the top of the column. Therefore, pre-column dispersion is almost negligible, while apparent efficiency is mostly affected by post-column dispersion [15]. The post-column system volume of most UHPLC systems ranges between 1 and 3 µL (typically detector cell of 0.5 to 2 µL and connecting tube of <1 µL).

Fig. 3 shows the peak widths measured at different gradient steepness. The measurements were performed at two gradient times (t_G) and two flow rates corresponding to four different in-

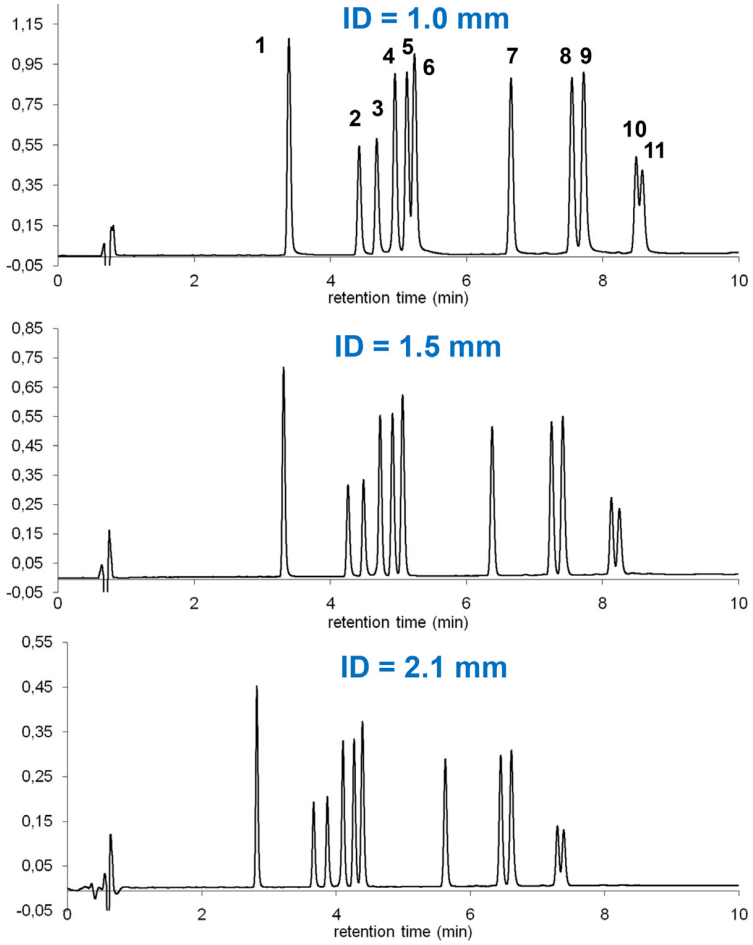

Fig. 4. Comparative chromatograms, obtained with 1.0 × 100 mm, 1.5 × 100 mm and 2.1 × 100 mm columns - packed with superficially porous C18 material - in gradient mode (standard UHPLC, Acquity H–Class). Mobile phase "A" is 0.1% formic acid in water, mobile phase "B" is 0.1% formic acid in acetonitrile. Linear gradient of 60 – 95% B at t_G = 10 min and at u_0 = 15 cm/min. Sample: CBDV (1), CBDA (2), CBGA (3), CBG (4), CBD (5), THCV (6), CBN (7), d9-THC (8), d8-THC (9), CBC (10) and THCA-A (11).

trinsic gradient steepness (b):

$$b = S \cdot \Delta\varphi \cdot \frac{t_0}{t_G} \quad (3)$$

where S is solute dependent parameter and $\Delta\varphi$ is the difference between the initial and final mobile phase composition expressed in volume fraction. Peak capacity (n_c) was determined according to the following equation:

$$n_c = 1 + \frac{t_G - t_0}{1.699 w_{1/2}} \quad (4)$$

At b = 0.025 (corresponding to high flow rate and long gradient time, Fig. 3A), the peak width ranged between $w_{1/2}$ = 0.032 and 0.072 min, corresponding to an average peak capacity of n_c = 223 on the 2.1 mm i.d. column. On the 1.5 mm i.d. column we measured $w_{1/2}$ = 0.036 and 0.076 min (average n_c = 205), while with the 1.0 mm i.d. column we obtained $w_{1/2}$ = 0.045 and 0.089 min (average n_c = 178). At b = 0.04 (corresponding to low flow rate and long gradient time, Fig. 3B), average peak capacity of 185, 163 and 145 were obtained with the 2.1, 1.5 and 1.0 mm i.d. columns, respectively. At b = 0.05 (corresponding to high flow rate and short gradient time, Fig. 3C), we measured peak capacities as n_c = 154 (2.1 mm), 141 (1.5 mm) and 119 (1.0 mm). And finally, with the steepest gradient (b = 0.08, corresponding to low flow rate and short gradient time, Fig. 3D) we observed n_c = 130 (2.1 mm), 115 (1.5 mm) and 95 (1.0 mm).

Compared to the 2.1 mm i.d. column, the 1.5 mm column performed about 7–11% less peak capacity, while the 1.0 mm i.d. column provided 20–27% lower peak capacity. The largest efficiency loss was observed with the steepest gradient, while the shallow gradients resulted in somewhat less significant post-column dispersion. This observation is in agreement with theory, since the gradient band compression effect is more pronounced with steep gradients and therefore thinner peaks are expected [14]. Fig. 4 shows a comparative example of cannabinoids mixture separations performed on 2.1, 1.5 and 1 mm i.d. columns at a gradient steepness of b = 0.08.

3.3. Studying LC-MS sensitivity

Besides the impact of reducing column diameter on efficiency, we have also evaluated its effect on MS sensitivity. For this part of the work, various doping agents were analysed in both ESI+ and ESI- conditions, at two different flow rates (low and high). The flow rates and injected volumes were geometrically adjusted between the columns of 2.1, 1.5 and 1 mm i.d. In addition, ESI conditions were optimized depending on the mobile phase flow rate. Fig. 5 highlights the behavior of one of the doping agents, namely chlorthalidone. Similarly, to what was previously described with UV detection, peak widths remain comparable between 2.1 and 1.5 mm i.d., but peaks are clearly broader on the 1 mm i.d. column. Due to the additional band broadening observed on the

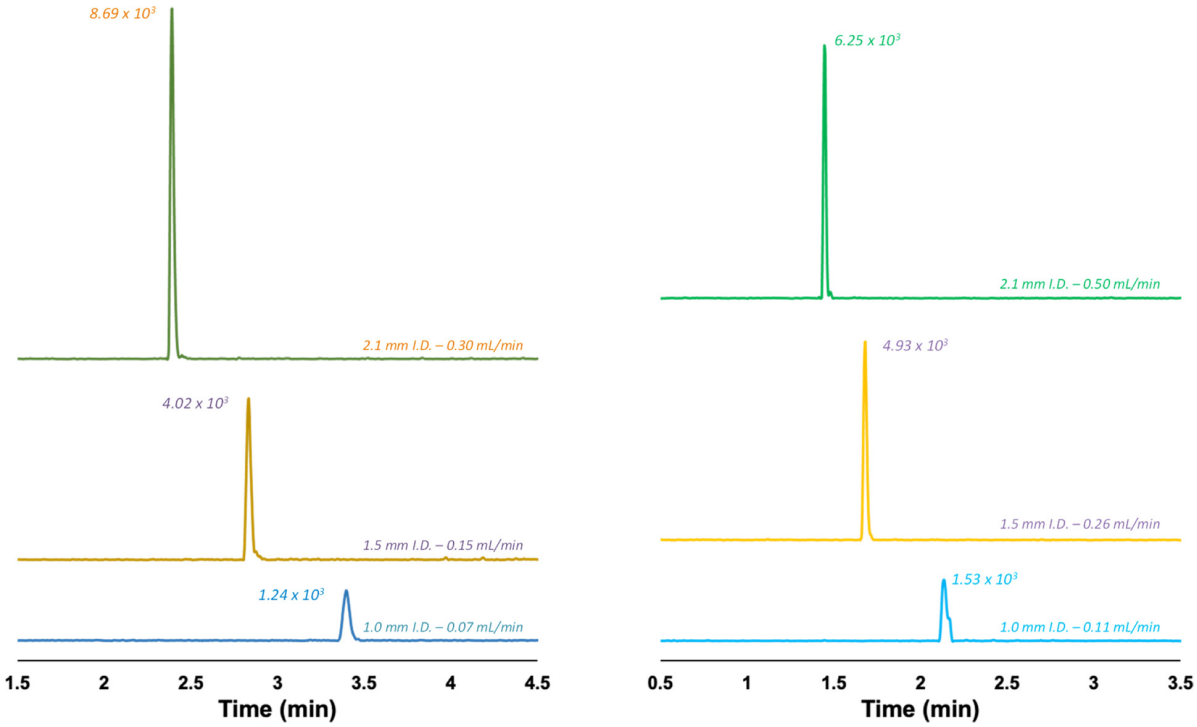

Fig. 5. LC-ESI-/MS/MS chromatograms obtained for chlorthalidone on 2.1, 1.5 and 1.0 mm i.d. at two different flow rates. Here, the flow rates and injected volumes were geometrically scaled with the column volumes.

1.5 vs. 2.1 mm i.d. column, the peak height was reduced by up to a factor 2 at the lowest flow rate. Importantly, the signal loss was much more pronounced on the 1 mm i.d. column (up to a factor 7 at low flow rate). For the 12 doping agents tested here, S/N was in average decreased by a factor 3.9 and 1.6 at low and high flow rate, respectively, when moving from a 2.1 to a 1.5 mm i.d. column. When modifying column i.d from 2.1 to 1 mm i.d., the average S/N values were reduced by 10.3 and 6.9 for high and low flow rate, respectively. Based on this data, it is probable that S/N would be less negatively impacted if a higher flow rate would be selected on the 2.1 mm i.d. column and geometrically transferred to other column dimensions.

Contrary to our expectations, the sensitivity decreased when using a smaller column i.d. (injected volume and flow rates were adjusted in direct proportion to the column volume). The only advantage when using 1.5 mm i.d. in LC-MS is to consume less sample (which can be important when analysing precious sample of limited volume), while having a limited impact on peak widths.

It is finally well known that the impact of mobile phase flow rate on ESI/MS sensitivity can be very different depending on the geometry of the ionization source (and the MS brand). Even if it has not been tested, it is possible that an improvement of sensitivity would be observed with the 1.5 vs. 2.1 mm i.d. column for a different MS device.

4. Conclusion

Our purpose was to find a compromise between 1.0 and 2.1 mm i.d. columns. Therefore, a prototype 1.5 mm i.d. column was prepared and compared to 2.1 and 1.0 mm i.d. columns packed with the same material (2.7 µm superficially porous 90 Å C18).

In isocratic mode, the loss of average apparent efficiency (plate numbers) was 6 – 10% and 12 – 31% with the 1.5 and 1.0 mm i.d. columns, respectively, compared to the 2.1 mm i.d. column.

In gradient mode, the loss of average peak capacity was about 7 – 11% with the 1.5 mm i.d. column and about 20 – 27% with the 1.0 mm i.d. column when compared to the 2.1 mm i.d. column. The largest efficiency loss was observed with the steepest gradient, while the shallow gradients resulted in somewhat less significant post-column dispersion.

When comparing the average S/N for the studied 12 doping agents, it decreased by a factor 3.9 and 1.6 at low and high flow rate, respectively, on the 1.5 mm i.d. column compared to the 2.1 mm one. When replacing the 2.1 mm i.d. column by the 1 mm i.d. one, the average S/N values were reduced by 10.3 and 6.9 for high and low flow rate, respectively.

Based on the results observed in this study, the new 1.5 mm i.d. column hardware seems to fit much better to current UHPLC instrumentation than the 1.0 mm i.d. column hardware. The loss in apparent efficiency and S/N ratio is much less important with the 1.5 mm id. column than with the 1.0 mm i.d. column. However, decreasing the extra-column volume of current instrumentation would resolve most of the problems related to the use of 1.0 mm i.d. columns.

Other benefits of the 1.5 mm i.d. hardware compared to the 2.1 mm i.d. columns are the lower sample and solvent consumptions. In case of properly scaled flow rates, the eluent consumption is about the half (factor 0.51) on the 1.5 mm i.d. column compared to a 2.1 mm i.d. column. In addition, probably less important frictional heating effects are expected due to the decreased operating flow rate.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Szabolcs Fekete: Writing - original draft, Methodology, Investigation, Validation. **Amarande Murisier:** Investigation, Validation, Writing - review & editing. **Gioacchino Luca Losacco:** Investigation, Validation, Writing - review & editing. **Jason Lawhorn:** Conceptualization, Resources. **Justin M. Godinho:** Conceptualization, Resources, Writing - review & editing. **Harry Ritchie:** Conceptualization, Resources. **Barry E. Boyes:** Conceptualization, Writing - review & editing. **Davy Guillarme:** Supervision, Writing - review & editing.

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IV.3 Chapter 2 – Application space for SFC in pharmaceutical drug discovery and development

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Chapter 2

APPLICATION SPACE FOR SFC IN PHARMACEUTICAL DRUG DISCOVERY AND DEVELOPMENT

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Abstract:

The purpose of this chapter is to provide a brief overview of the past, present and future applications of supercritical fluid chromatography (SFC) in pharmaceutical drug discovery and development.

In the first part of this chapter, various examples are provided, showing how SFC has been used in the past. Originally employed for the analysis of apolar compounds, SFC rapidly became an interesting tool to substitute NPLC for chiral separations, due to its higher efficiency, faster analysis times, reduced environmental impact and lower costs. Developments in the early 2000s begun exploring the usefulness of SFC also for achiral analyses, but the instrumentation available was not capable to meet the well-established robustness and sensitivity criteria used in liquid and gas chromatography, thus discouraging its implementation in pharmaceutical analysis. However, the introduction of ultra high performance supercritical fluid chromatography (UHPSFC) systems from 2012 has played a key role in refueling interest in this technique. With better instrumentation, UHPSFC demonstrated very promising results in the pharma industry also in the context of achiral separations, from the impurity profiling of synthetic APIs to its implementation in more challenging contexts, such as peptide analysis.

In the second part of the chapter, considerations on the SFC stationary and mobile phases, its coupling to detectors (especially MS) and, finally, performance in the context of method validation are discussed. The recent evolution of SFC has depicted an image of this technique being efficient, fast, complementary to liquid chromatography (LC) and finally able to satisfy different validation criteria. Finally, an interesting perspective for SFC is

linked to its implementation in multidimensional systems, with the aim to expand the chromatographic selectivity.

Keywords: Supercritical fluid chromatography; Impurity profiling; Method validation; High throughput; drug discovery; drug development

1. Introduction

Drug discovery/development is an expensive and tedious process, which requires approximately 12–15 years to produce a new drug and an estimated $2.6 billion to reach the market (1). When searching for a new molecule that can modulate disease process during the initial phase of the drug development process (drug discovery phase), it is important to keep in mind that the available chemical diversity is incredibly large. Indeed, the number of small molecules with a molecular weight up to 500 and containing only the most common atoms has been estimated at 10^{60} (2). In another contribution, it has been shown that the number of possible peptides having 62 amino acids and composed of the 20 natural amino acids has been estimated at 10^{80} (3). Among this available chemical space, only a small fraction has thus far been explored, and allows to interact with and perturb the function of given biological molecules. It is also important to keep in mind that there is no information shared between companies about inefficient compounds, making the work even more difficult for pharmaceutical companies. Therefore, the procedure required in the pharmaceutical industry to find appropriate compounds is remarkable. During the drug discovery process, it is indeed required to screen millions of potential compounds (this number remains insignificant compared with the total number of possible small organic molecules) each year against a range of targets, and even then, success is not guaranteed. To help selecting the compounds with suitable drug likeness, before the substance is even synthesized and tested, some rules have been developed based on the physico-chemical properties of the molecules as an approach to reduce attrition in drug discovery and development. The most famous one is the Lipinski's rule of five, which covers the number of hydrophilic groups (no more than 5 hydrogen bond donors and 10 hydrogen bond

acceptors), molecular weight (less than 500 Da) and hydrophobicity (log P lower than 5) (4). However, simple rules are not always perfectly accurate and may unnecessarily limit the chemical space to search. Therefore, many pharmaceuticals are also now screening compounds that have properties outside this chemical space, and many best-selling drugs have features that cause them to score low on various drug likeness indices (5,6). Among the exceptions, most of the favourable ones to the Lipinski's rule of five occur among natural products. Indeed, natural products are universally recognized to contribute valuable chemical diversity to the design of molecular screening libraries as reported elsewhere (7,8), despite some difficulty to access renewable sources of plant-derived products (9). To better understand the chemical space covered by synthetic molecules and natural products, Figure 1 (10) highlights the obvious differences between three different groups of compounds, namely known natural products, readily obtainable natural products, and drug molecules (11), through the construction of PCA plots.

Despite significant advances in technology, the drug discovery process remains expensive and tedious. With the existence of such very large libraries of synthetic or natural compounds having diverse/heterogenous chemical properties, there is a need to develop innovative analytical strategies, mostly based on chromatography, able to produce a generic, fast, automated, and high-throughput screening method adapted to the largest number of compounds in the pharmaceutical research and development (R&D) environment, to reduce the attrition rate (particularly the late stage NCEs costly failures). The main application areas of chromatography in modern drug discovery are summarized below (12). First, some reliable and generic chromatographic methods have to be developed to check identity and amount of impurities in new potential lead compounds

from natural and/or synthetic sources. To meet these objectives, it is required to develop open-access, fully automated LC-UV and LC-MS systems that can be used by chemists (13). In addition, there is a need to chromatographically purify a sufficient amount of compounds (this is particularly difficult when compounds contain a chiral centre and the toxicity profile of each enantiomer has to be assessed) for pharmacokinetics/pharmacodynamics studies.

From an analytical point of view, reversed phase liquid chromatography (RPLC) coupled with UV or mass spectrometry (MS) detection is considered as the gold standard analytical strategy in the pharmaceutical industry. RPLC can indeed be applied to any compound having log P comprised between -1 and 6 (this lipophilicity range fits very well with the Lipinski's rule of five) as highlighted in Figure 2. In RPLC, an apolar stationary phase is used (i.e. C4, C8, C18, phenyl), while the mobile phase is composed of a mixture of buffered water and polar organic solvents (i.e. acetonitrile, methanol, tetrahydrofuran). The retention mechanism of RPLC is mostly based on hydrophobic interactions, but other mechanisms can also take place depending on the mobile phase conditions and chemical nature of the stationary phase (i.e. shape recognition, ionic interactions, π- π interactions). The success of RPLC is mostly due to its versatility, easiness to use, robustness, easy hyphenation to MS and widespread use in the pharmaceutical industry.

However, when considering compounds outside this lipophilicity range, alternative strategies are required. Among them, we can cite the use of normal phase liquid chromatography (NPLC) for the analysis of compounds having log P values ranging from 2 to more than 10 (i.e. halofantrine, cyclosporine A, propafenone, moclobemide, orlistat, amiodarone…), which are too strongly retained under RPLC conditions. In NPLC, the

stationary phase is polar (i.e. bare silica, amino, diol, cyano…), while the mobile phase is mostly apolar and exclusively composed or organic solvents (i.e. hexane, ether, ethyl acetate, isopropanol…). In NPLC, the retention mechanism is solely based on polar interactions (i.e. dipole-dipole, H-bond). However, the use of NPLC is associated with a significant number of drawbacks, including the use of highly toxic and expensive solvents, a poor applicability to ionisable compounds, a very limited compatibility with MS detection, and restricted robustness (14). On the other hand, when hydrophilic compounds ($\log P < 0$) have to be analysed (i.e. streptomycin, acarbose, lysinorile, ibandronic acid, …), retention is clearly insufficient in RPLC mode and alternative strategies have to be considered. Ion pairing reversed phase liquid chromatographic (IP-RPLC) was used in the 90's and 2000's but has now been progressively replaced by hydrophilic interaction chromatography (HILIC) – see figure 2. In HILIC, a polar protic stationary phase is used (i.e. bare silica, amide, diol, amino, zwitterionic…), while the mobile phase is composed of a small proportion of buffered water (5 to 40%) mixed with an aprotic solvent (generally acetonitrile) (15). The retention mechanism is quite complex, since it is a mix of hydrophilic partitioning (partition between a water enriched layer located at the surface of the stationary phase and the remaining mobile phase mostly composed of acetonitrile), and polar interactions (mostly ionic and H-bond) (16). HILIC provides a suitable retention for hydrophilic compounds and excellent MS compatibility. However, it requires long equilibration times and is not as robust and versatile as RPLC.

As previously exposed, HILIC, RPLC and NPLC modes allow covering the whole range of possible molecules that can be found in drug discovery/drug development, whatever their physico-chemical properties. However, HILIC and NPLC modes suffer from several

limitations and are clearly not as powerful as RPLC. For this reason, supercritical fluid chromatography (SFC) has been suggested as a suitable alternative strategy to cover the widest possible range of molecules (see figure 2) (17). In SFC, any type of stationary phase can be considered (i.e. polar, apolar, aromatic). The mobile phase is mostly composed of supercritical CO_2 (CO_2 is used above its critical point, temperature > 31°C and pressure > 72 bar) mixed with any type of organic solvent (from hexane to methanol) and additives (water, salts…). The possibility to use almost any type of stationary phases and mobile phases make SFC a highly versatile technique. However, in practice, a polar stationary phase is commonly used, and mobile phase often consists of a mixture of CO_2, methanol and additives. Under such conditions, the retention mechanism is mostly based on polar interactions (dipole-dipole, ionic interactions and H-bond), similar to what is found in NPLC. It is also important to notice that SFC can be considered as a green strategy (mobile phase mostly composed of supercritical CO_2), it offers excellent kinetic performance (low viscosity) and a strong potential for chiral separations and purifications. As already demonstrated about 15 years ago (18), SFC can be considered today as a highly valuable strategy for high throughput screening of large and diverse library of pharmaceutically relevant compounds, representative of what can be found in drug discovery.

2. Discussion

2.1 Historical overview of SFC applications

2.1.1 1980-2000: SFC for the analysis of apolar substances and chiral drugs

In its early days, SFC has been identified as a suitable technique for the analysis of hydrophobic compounds (19,20). The first applications involving SFC implemented pure supercritical CO_2 as the mobile phase. Although other molecules have been tested in their

supercritical state, the choice fell quite rapidly on CO_2 due to the easiness in reaching its critical point. However, the polarity of supercritical CO_2 was estimated to be similar to that of apolar organic solvents, such as *n*-hexane or toulene according to the pressure applied. Thus, SFC began being implemented for the analysis of apolar substances, such as lipids or hydrocarbons in petroleum samples (21,22). The need to expand the applicability range became rapidly relevant, therefore the addition of polar liquid co-solvents, miscible with supercritical CO_2, was tested. Among the different solutions, small alcohols (e.g. methanol, ethanol, isopropanol, etc.) were often selected (23–25). Their use allowed to greatly increase the polarity of the SFC mobile phase, thus allowing the analysis of compounds with a limited hydrophobic character (25). More importantly, SFC began to be considered as an attractive alternative to liquid chromatography (LC). Due to the similarity in the polarity of the mobile phase, SFC was tested for the analysis of compounds normally assessed using NPLC conditions. Consequently, SFC began to be implemented mainly in the context of chiral analyses (26,27). Chiral stationary phases, previously developed for LC, indeed demonstrated good performance also under SFC conditions. On the other hand, a general reduction in the analysis time was obtained in SFC vs. NPLC (Fig. 3), due to the possibility to reach higher flow-rates thanks to the reduced backpressures generated under SFC (27). Moreover, the replacement of the organic solvents commonly employed in NPLC (i.e. *n*-hexane, *n*-heptane, dichloromethane, etc.) with a mobile phase rich in CO_2 gave different advantages to the analytical laboratories: reduced environmental impact and lower cost. Furthermore, SFC showed an interesting potential when purifying compounds, as the supercritical CO_2 would simply shift in its gaseous state when fractions are collected, thus leaving the purified sample with only minimal percentages of the liquid co-solvent.

2.1.2 2000-2010: SFC as an alternative to RPLC for achiral applications

Starting from the early 2000s, more efforts were put to further develop SFC. The first stationary phases dedicated to SFC were developed and optimized, using polar selectors with some basic properties, such as 2-ethylpyridine (2-EP) or 4-ethylpyridine (4-EP), and LC columns were also continuously used in SFC (28–30). Innovative mobile phase conditions were also developed, in particular with the use of acidic and basic additives. The aim was to improve the peak shape (peak width and tailing). Hence, the use of acidic or basic additives began to diffuse among laboratories (31). Moreover, higher modifier percentages were being tested (up to 25-40%). With a more abundant presence of the polar co-solvent, SFC started to increase its appeal also for achiral applications involving the analysis of moderately polar compounds with a log P value as low as -1. This evolution makes SFC compatible with the impurity profiling in the pharmaceutical world, for the analysis of small synthetic API and their related impurities (e.g. beta-blockers, local anaesthetics, benzodiazepines, etc.), as shown in Fig. 4 (32,33). SFC soon demonstrated its complementarity to RPLC providing a different, and in some cases even better separation profile. Indeed, several publications reported the high interest of SFC for screening method and purification of R&D batches. A first example proposed a three-steps strategy in the context of drug discovery: a pre-analytical run enabled to assess the separation condition before moving to the preparative step. Afterwards, a post-analytical step was done to verify compound purity before further investigations (34). Each run had a maximal time of 5 minutes with a purification rate above 90 % to have a really fast and efficient overall process to support medicinal chemistry. In the same way, another work proposed a generic method using 2-EP stationary phase for high throughput analysis and preparative steps

(35). Despite good results regarding method orthogonality and the purity level at the end of the process (above 95 %), the method was considered as not generic enough for the whole range of compound functionalities observed within discovery chemistry environment.

Such approach was also proposed in the field of chiral compounds (36). They successfully implemented a generic high throughput chiral separation to speed up method development for providing fast and reliable results to the chemists. SFC provided faster separation and better resolution than existing methods (NPLC and RPLC). Nevertheless, several drawbacks were described for the implementation of this strategy in the laboratory: (i) the analysts were not familiar with SFC and training was not easy due to instrumentation complexity (in the beginning of the 2000's); (ii) the cost of the instrumentation was quite high in comparison with existing technique; (iii) the main issue was the disinterest of instrumentation manufacturer leading to non-optimal design, poor robustness and low quality technical follow-up for exiting systems.

In conclusion, SFC struggled to find its space in the analytical portfolio of pharmaceutical laboratories, as it was not able to provide satisfactory robustness and quantitative performance. There are several reasons for the poor repeatability of SFC analytical methods: super/subcritical fluids are much more compressible than liquids, thus they are more exposed to the generation of density gradients when high backpressures are reached (37). These density gradients are known to cause localized shifts in the elution strength of the mobile phase, especially in those regions where the supercritical CO_2 becomes denser. As these shifts in the mobile phase's elution power cannot be controlled, SFC methods have a high probability of experiencing a limited retention times repeatability. Moreover,

the sensitivity observed in SFC with a UV detector is often strongly reduced compared to LC-UV (38). Indeed, the instrumentation available at the time did not always guarantee an optimal mixing between the supercritical CO_2 and the liquid modifier, due to issues in the variation of the refractive index. Finally, it also showed difficulties in keeping the mobile phase in its super/subcritical state, especially when increasing percentages of co-solvent were utilized. All these issues contributed to generate a high UV background noise, thus making impossible for analysts to reach and respect the more stringent detection levels set by regulatory agencies. Finally, the introduction in the early 2000s of the first UHPLC systems, in combination with the development and systematic use of columns with sub-2 μm silica particles contributed to make SFC a less attractive technique in analytical laboratories.

2.1.3 2010-2014: Transition from SFC to UHPSFC

The development and subsequent release on the market of a new generation of SFC instruments in the early 2010s proved to be fundamental in establishing a future to this technique. These novel systems are mainly characterized by a much lower extra-column and gradient delay volumes, as well as an ability to reach and sustain backpressure values as high as 400-660 bar. Their release enabled SFC to accomplish a further evolution step, described by some as ultra-high performance SFC (UHPSFC) (39). UHPSFC was able to compete with UHPLC in terms of kinetic performance, regaining interest from different analytical laboratories. In parallel to the instrumental developments, column manufacturers produced a great effort in releasing a new and improved generation of SFC stationary phases, using sub-2 μm (hybrid) silica particles, with a special focus in obtaining selectors providing complementary properties and, at the same time, improved peak shapes for basic

and acidic compounds (40). However, one of the most important breakthroughs was the understanding of the role of different additives in tuning the elution strength and properties of the mobile phase. Not only the use of MS-compatible salts such as ammonium formate (AmF) and ammonium acetate (AmAc) started to become more widespread, but it was also found how the addition of water, at low percentages in the liquid co-solvent, was vital in obtaining an impressive boost in the elution power of the SFC mobile phase regarding polar substances (41,42). With the addition of water in the mobile phase, SFC experienced a further expansion of its applications, now focusing on more polar analytes (Fig. 5) (41,43,44). This allows SFC to be considered as an alternative not only to NPLC and RPLC, but also to HILIC. Furthermore, the analysis of substances (such as doping agents) in biological matrices (urine, plasma…) became a reality with SFC (45). The advantages of the new UHPSFC instrumentation also allowed a much-improved performance of SFC in routine laboratories. Thanks to the better design of the back-pressure regulator module, as well as of a better technical understanding in the handling of a super/subcritical fluid, SFC began providing more robust analyses (46). Furthermore, since numerous SFC methods use a proportion of organic modifier up to 45-50%, the mobile phase suffered less from compressibility, thus reducing the extent of the density gradient phenomenon. All this helped in establishing a satisfactory reproducibility of retention times in UHPSFC, with this technique eventually starting to demonstrate good results also in the context of method validation (47). Finally, the new instrumental design enabled SFC to get closer to LC in terms of UV sensibility, with comparable performance.

2.1.4 2015-present days: SFC for highly polar compounds

Until now, the discussion revolved on the analysis of small molecules with a modest polar character, but SFC was also tested for more challenging molecules, such as peptides (48). Preliminary experiments indicated of the possibilities offered by SFC for analysing highly complex compounds, as well as samples possessing a high polarity. However, to verify if SFC would be able to provide good performance, the analytical conditions have to be adjusted. A clear example is given by the development of enhanced fluidity liquid chromatography (EFLC). In EFLC, CO_2 is not anymore the main component of the mobile phase, it is rather employed to reduce the viscosity of the mobile phase that is mainly composed of a liquid (49). The nature of the liquid itself is different: as the supercritical CO_2 is now less present, a higher percentage of water can be considered (up to 20-25%) mixed with methanol. These extreme changes were successfully employed in the analysis of different biomolecules (e.g. polysaccharides, nucleobases, proteins, etc.) with a technique derived from SFC, in an attempt to reach a connecting point between liquid and supercritical fluid chromatography (50,51). On the other hand, EFLC is poorly adapted to mildly polar or apolar analytes. Thus, some attempts were made to find conditions enabling SFC to successfully analyse, at the same time, apolar and polar substances. Some laboratories decided to verify the capability of an innovative SFC gradient called Unified Chromatography (UC) (52). With UC, the analysis would begin with a predominant percentage of CO_2 (between 98-95%), as commonly done in SFC applications. However, the gradient would reach percentages up to 100%. The mobile phase would therefore completely transition from a supercritical to a liquid state within a single analytical run. The use of UC gradients was often combined with the addition of water in the mobile phase: although it is not possible to reach the high percentages seen with EFLC gradients

due to the predominant presence of CO_2 in UC-type gradients, percentages up to 5-7% of water have become more common in the works of different research groups. Moreover, the increased amount of water available in the co-solvent allowed an increase in the additives and salts concentrations, reaching in some cases values as high as 150 mM (53). With such optimized conditions, SFC started to be successfully utilized in areas of applications which were almost impossible to consider in the past: from metabolomics (54,55) to peptides (56) and proteins analysis. Indeed, SFC is currently experiencing a growing interest and use in fields where HILIC or ion exchange chromatography were the only choices at a disposal to chromatographers. In addition, UC-type gradients still give the possibility to analyze mildly polar or apolar substances. As shown elsewhere (Figure 6), UC-type gradients can still provide the necessary retention for apolar compounds such as lipids or fat-soluble vitamins, and simultaneously generate the needed elution power for highly polar compounds that are strongly retained by the stationary phase (52,53). Moreover, as most of the additives and salts chosen in SFC are fully MS-compatible, the hyphenation with MS is still possible, with UC-type gradients. Such approach was recently experienced in the field of biopharmaceuticals drug discovery. A first paper reported the interest of SFC (almost UC) for the separation of crude mixture synthetic peptides of various sequence lengths (57). Several stationary phases and additives (see section 2.2) were tested to propose generic method(s) adapted to peptide length and conformation. This study can be considered as a proof of concept, showing the interest of SFC regarding unique selectivity and fastness. However, HPLC still outperformed SFC for peptides with long sequences. Another paper proposed SFC as a purification method for peptides (58). Their results also highlighted the interest of SFC for peptide purification at the low preparative scale used in

the field of drug discovery. These two examples highlighted the potential of SFC extended to larger compound, which represented a large proportion of the new therapeutic molecules.

2.2 Considerations on SFC as an analytical tool in drug discovery and development

2.2.1 Mobile phase composition

As previously discussed, SFC underwent several evolutions in its history. Each time, one of the main factors which allowed to overcome its limits was the nature of the mobile phase. From the use of pure supercritical CO_2 in the 1980s and early 1990s to a ternary mixture of CO_2, methanol and water, with additives and salts at relatively high concentrations, the flexibility that SFC provides by simply modifying the composition of its mobile phase is a value that no LC technique can provide. This can be accomplished also without changing the nature of the stationary phase, while in LC it is mandatory to change firstly the properties of the stationary phase (and, consequently, the mobile phase) to obtain good results with either apolar or polar analytes. Thus, it is fairly simple to understand how SFC could provide to scientists more solutions than LC. This incredible potential is provided by mixing, at different compositions, the four main components in the mobile phase (supercritical CO_2, organic modifier, water and additives/salts) (42,59). If apolar, or water-unstable compounds are analysed, the mobile phase should be predominantly composed of supercritical CO_2, with moderate percentages of organic co-solvent (60). The increase in the mobile phase elution strength given by the addition of water or salts ensures the successful elution of highly polar substances. Additives and, more specifically, water have become increasingly important in the context of method development in SFC, as they allow

to achieve suitable performance for polar and highly polar compounds. As recently demonstrated by an increasing number of papers, the use of water-rich mobile phase with additives of various nature in SFC has enabled the analysis of challenging samples such as nucleosides, amino acids, sugars and, finally, even peptides and small proteins (51,56,61–63). More details on the performance of SFC-based analytical methods for biomolecules and highly polar compounds can be found in Chapters 4 and 9.

2.2.2 Impact of the stationary phase

As mentioned in the previous section, an important amount of work has been put in optimizing the SFC mobile phase conditions. Nonetheless, the performance of any chromatographic method, including SFC, remains linked to the chemical nature of the stationary phase. Unlike to what has been witnessed in RPLC with the proliferation of the C18 stationary phase, the column screening phase is still an extremely relevant part of any method development process for SFC. Different chemistries have been developed specifically for this technique, for example selectors with a basic moiety, such as 2-ethylpyridine (2-EP) or diethylamine (DEA) and 2-picolylamine (2-PIC). In addition, LC stationary phases can be utilized as well, and diol-based, bare silica columns, aromatic stationary phases such as pentafluorophenyl (PFP), and even non-endcapped C18 columns have proven themselves useful in SFC. As they all demonstrate different chemical nature, they guarantee different selectivity. However, a closer look highlights some common features among most of SFC columns: the majority of SFC columns possess some degree of polarity, as well as the ability to generate different interactions, such as H-bonds, π-π interactions, dipole-dipole. More details on the characteristics and main properties of the SFC stationary phases can be found in Chapter 3. As explained above, the selection of the

suitable SFC stationary phase remains an important step of method optimization. In this context, the selection of an orthogonal set of stationary phases could help method development (64). In the present study, API and degradation products of confidential development compounds were used to screen the selected stationary phases before method optimization. This strategy helped to select suitable conditions for the analytical separation before scaling-up to purification. In is important to notice that the analytical SFC method was further used to evaluate fraction purity.

In section 2.1.4, it was highlighted how the choice of the gradient profile has become an important factor today. There has been a rising trend to use unorthodox gradients in which either the supercritical CO_2 would be not the main mobile phase component, as in the case of EFLC, or in performing the shift from supercritical to liquid conditions within a single analytical run, such as for UC-type gradients. These innovative gradients are being increasingly used in SFC for the analysis of biomolecules and compounds present in biological fluids, but there are some considerations that need to be done. In both cases, EFLC and UC-type gradients foresee the use of high percentages of modifier in the mobile phase, from 50% up to 80-100%. As the amount of co-solvent increases, column backpressure also drastically increases, which become more important when using columns packed with sub-2 μm particles. This can be problematic when using high flow rate, as the current generation of UHPSFC systems do not sustain backpressures higher than 400-660 bar. UC-type gradients tend to suffer more compared to EFLC, as high flow-rates are used in UC conditions, to ensure good kinetic performance, with the low percentages of modifier employed at the beginning of the gradient. To address this issue, different strategies can be applied. One potential solution is to increase column temperature

to 50-70°Cto reduce mobile phase viscosity when high co-solvent percentages are reached, thus lowering the backpressures generated with a sub-2 µm stationary phase. Moreover, higher temperatures have demonstrated to improve the C-term of the van Deemter curves with a mobile phase predominant in the liquid modifier, following a similar behaviour shared by liquid chromatography (65). A second strategy is to use a different morphology of silica particles, such as superficially porous particles (SPP) (66,67). The introduction of sub-3µm SPP was of great help to reduce the elevated backpressures generated by the sub-2 µm fully porous particles, without experiencing major loss in the kinetic performance, however their use in SFC is still not so widespread. A generic SFC gradient with a co-solvent percentage lower than 40-50% does not generate elevated backpressures with a sub-2 µm stationary phase at relatively high flow-rates, thus there is limited interest for SPP in SFC. On the other hand, as EFLC and UC-type gradients make their way, the interest of SPP can experience a regain of interest, as they can provide lower backpressures and almost identical kinetic performance.

2.2.3 Choice of the injection solvent

The nature of the sample diluent could strongly impact peak shapes in SFC. Due to the peculiar retention and elution mechanism in SFC, it is important to choose an injection solvent which does not interact too much with the stationary phase. The most employed solvents used as sample diluent in the earliest applications were hexane, heptane, dichloromethane or methyl ter-butyl ether (MTBE). Those are aprotic, apolar solvents which have immediately provided good chromatographic performance, with a minimized peak distortion phenomenon (39). Unfortunately, they also strongly limited the applicability range of SFC, since it would only be possible to solubilize samples with

limited polarity. Thus, several efforts were put in identifying alternative solvents, which would ensure sufficient solubility even for more polar analytes, without compromising the peak shape and symmetry. Mixtures of the previously mentioned solvents with small alcohols such as isopropanol, ethanol and, more rarely, methanol started to become more systematically employed. In addition, acetonitrile (ACN) arose as one of the most promising alternatives. ACN is, indeed, a good solvent not only for apolar compounds, but also for mildly polar substances such as synthetic drugs. It is perfectly miscible with methanol or water and, more importantly, it does not strongly interact with the stationary phase. It, therefore, guarantees good peak shapes and solubility of a wide range of molecules (68).

On the other hand, acetonitrile cannot always be successfully employed with more polar analytes or when biological matrices are considered. However, as SFC began its shift towards the use of increasingly higher percentages of co-solvent, it became possible to employ even more polar injection solvent. More importantly, the widespread use of EFLC and UC-type gradients seems to have enabled the use of methanol, or even water as component of the sample diluent. In few cases, pure water was used without any deterioration of the chromatographic parameters (56). Indeed, with compounds strongly retained by the column, which need high percentages of liquid modifier to elute, the impact of the sample diluent nature becomes less important. However, if analytes do not show a strong affinity for the stationary phase, they will inevitably suffer from peak distortion if protic solvents are used. This phenomenon is particularly important when compounds with both poor and strong retention properties need to be analysed simultaneously. In that case, it was demonstrated that a mixture of an aprotic with a protic solvent could be an attractive

strategy. In this example (53), a mixture of ACN and water with a 50:50 *v/v* ratio was fundamental in ensuring the best peak shape for analysing simultaneously compounds with very different properties. More details on the strategy to choose the injection solvent for SFC analyses can be found in Chapters 7 and 8.

2.2.4 Hyphenation to mass spectrometer detectors

Similar to LC, SFC can also be coupled to various detectors. Ultraviolet (UV) detector is probably the most widely used, but there has been a constant trend to use other detectors. Mass spectrometers (MS) are those attracting a lot of interest from researchers and analytical laboratories; thus, instrument providers began developing some technical solutions to easily hyphenate SFC with MS. Each hyphenation strategy brings its own advantages and disadvantages (more information can be found in Chapter 4). Indeed, due to the super/subcritical nature of the SFC mobile phase, system developers had to come up with a solution to couple the MS and the back-pressure regulator module, as well as to handle the decompression of supercritical CO_2 in the connection tubing between SFC and ionization chamber (69,70). In this last segment, as the decompression phenomenon causes the CO_2 to pass in its gaseous state with a lowering of the temperature due to the endothermic nature of this process, there have been several precipitation issues as the mobile phase would not be able to solubilize anymore the samples. The addition of a make-up pump, delivering an additional solvent (generally MeOH), helped resolving this issue. The make-up pump demonstrated to be essential for SFC-MS operation, more importantly when the developed SFC method did not use high percentages of co-solvent (up to 20-30%). On the other hand, its role might need a re-evaluation in the case of EFLC and UC-type gradients. As previously discussed, under these conditions, the amount of liquid

modifier reached elevated percentages (>70-80%), becoming the predominant element of the mobile phase. Quite logically, it can be expected that the current SFC-MS interfaces might not be the best ones with these conditions. More specifically, the use of a make-up pump might become irrelevant when high modifier percentages are reached, thus avoiding the precipitation issues previously discussed. A possible solution would be to employ a flow-rate gradient for the make-up pump, to have the needed make-up solvent at the beginning of the analysis as the co-solvent levels are quite low and its reduced presence towards the end of the gradient profile. However, due to the limited research being done on this topic, it becomes hard to draw some definite conclusions.

2.2.5. Quantitative performance of SFC

Analytical method validation is a major concern for analysts, especially in the pharmaceutical field. Method validation is required by the guidelines from the step of (pre)-clinical studies (see Chapter 7 and Chapter 11). Obviously, method validation as described by ICH Q2 R1 (71) is not required and adapted for drug discovery environment, but could be fully advised for pharmacokinetic studies. Furthermore, the assessment of SFC quantitative performance by means of method validation is a major asset to confirm the potential and the reliability of this technique.

As mentioned above, SFC suffered during many years from a bad reputation of unreliable and not robust chromatographic technique. Since the resurgence of SFC in the beginning of 2010's and the launch of new instrumentations, several studies were performed to deeply demonstrate the quantitative performance of this technique (72). Firstly, the technical improvements of new SFC were clearly highlighted as beneficial to improve UV sensitivity, injection reproducibility and overall method robustness (73). More recently,

SFC-MS/MS was proposed as an alternative technique in the field of the nitrosamines crisis (74). Indeed, the method was developed following the principles of analytical lifecycle management through robust optimization strategy. This study means that SFC is able to face the analytical challenges and the regulatory requirements. Finally, besides the demonstration of SFC quantitative performance by means of method validation, the evaluation of SFC method reproducibility was also performed thanks to inter-laboratory study (75). This study demonstrated the third level of method precision, i.e. reproducibility. The results highlighted that SFC method was successfully transferred in 18 laboratories with reproducibility RSD values better than values reported for LC impurities determination. To summarize, these studies confirmed the quantitative performance and robustness of modern SFC. It means that this technique is reliable and should be used in the field of drug discovery to benefit from its versatility and efficiency.

In the specific topic of drug discovery and development, it is interesting to focus on degradation and impurity analysis. Indeed, after a first drug screening, drug candidates should be characterized to further proceed to pharmacological assay (76). In this context, an interesting paper reports the comparison between UHPLC and SFC for the impurity profiling of drug candidates (77). At this stage of drug development, impurity profiling method should be able to identify the API, evaluate the purity of the main component and identify all impurities with an estimated concentration above a pre-defined threshold. In this context, the analytical method should be (i) selective to propose a large separation between the API and impurities; (ii) precise and (iii) sensitive to be able to detect very low quantity of impurities. Based on previous references, SFC with UV and/or MS detection can be proposed as an interesting analytical technique to face these challenges.

3. Conclusion & Perspectives

As highlighted in this chapter, SFC can be considered today as a reliable analytical strategy that should be more used widely in the coming years in the pharmaceutical drug discovery and development. Even if HPLC is still the gold standard in the pharmaceutical industry, SFC offers a number of significant advantages that need to be considered. First of all, SFC has the potential to replace RPLC, NPLC and HILIC, for the analysis of highly hydrophilic to lipophilic substances, thanks to the use of adapted mobile phase and stationary phase conditions (i.e. EFLC, UC). It also offers orthogonal selectivity compared to RPLC, due to the very different retention mechanism (hydrophobic interactions in RPLC vs. polar interactions in SFC). In addition, modern SFC (also known as UHPSFC) offers excellent kinetic performance and ultra-fast separations (important for HTS application), thanks to the use of columns packed with sub-2µm particles and the low mobile phase viscosity. SFC is also fully compatible with MS and various interfaces are now commercially available (69,70). SFC should also be considered as a greener alternative to liquid chromatography, due to the fact that mobile phase is mostly composed of CO_2, which has a significant economic and ecological value. SFC is also considered as one of the most powerful strategy for enantioseparation (a large majority of drugs are chiral) and is particularly beneficial at the preparative scale (CO_2 can be easily eliminated). Last, it has been recently demonstrated that the robustness and quantitative performance (method validation, inter-laboratory studies) obtained with modern SFC system were comparable to that observed in RPLC, which is an important feature for pharmaceutical analysis.

However, it is also important to keep in mind that some progresses still need to be make in SFC to further expand its use in the pharmaceutical industry. First, more powerful SFC

systems are required, offering higher upper pressure limit (up to 1000 bar), and reduced system volumes (extra-column volume and dwell volume) (78). Secondly, even if fundamental studies are published in SFC for more than 30 years, we still have to understand how additives (i.e. water, salts) and even methanol could adsorb at the surface of the polar stationary phase, thus explaining some unexpected results (79). Method transfer (from SFC to UHPSFC, and from analytical to preparative SFC) is also something more difficult to handle in SFC *vs.* RPLC and certainly also deserves some special interest (80).

In the last few years, there has been more and more interest towards the use of multidimensional LC (2D-LC) systems to further improve the overall performance in chromatography (i.e. higher peak capacity, enhanced selectivity, improved MS compatibility…) (81). In our opinion, the pioneering work of Venkatramani *et al.*, highlighting the combination of achiral RPLC and chiral SFC is particularly interesting for pharmaceutical analysis (82), even if the experimental setup is still complex and far from being used in routine laboratory. A combination of apolar SFC column and polar SFC column could also be a valuable approach to improve analyte coverage (from hydrophilic to lipophilic ones).

Figure 1: Scatter plots of the second PC against the first PC for the reference data sets based on 17 relevant physicochemical properties. The figure shows the data points of the known NPs, readily obtainable NPs and approved drugs. Adapted with permission from (83).

Figure 2: Lipophilicity range covered by different chromatographic techniques (NPLC, RPLC, HILIC, SFC and water-rich SFC

Figure 3: Comparison of HPLC and SFC chiral separation of CBZ-norvaline enantiomers on Chirobiotic R (left) and T (right). Reprinted, with permission, from (84).

Figure 4: Chromatograms of a complex pharmaceutical standard compound mixture on cyanopropyl silica column under SFC conditions. Reprinted, with permission, from (33).

Figure 5: SFC chromatograms of a real-life urine sample of a patient after administration of norketamine, before (black) and after 0-24h (pink), 24-48h (blue), 48-72h (brown). Adapted, with permission from (85).

Figure 6: Simultaneous analysis of triconasoic acid and raffinose in SFC/UC conditions. Reprinted, with permission, from (53).

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Figure 1

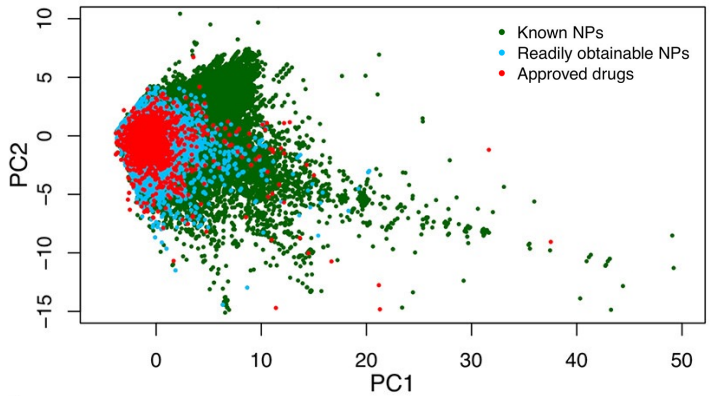


Figure 2

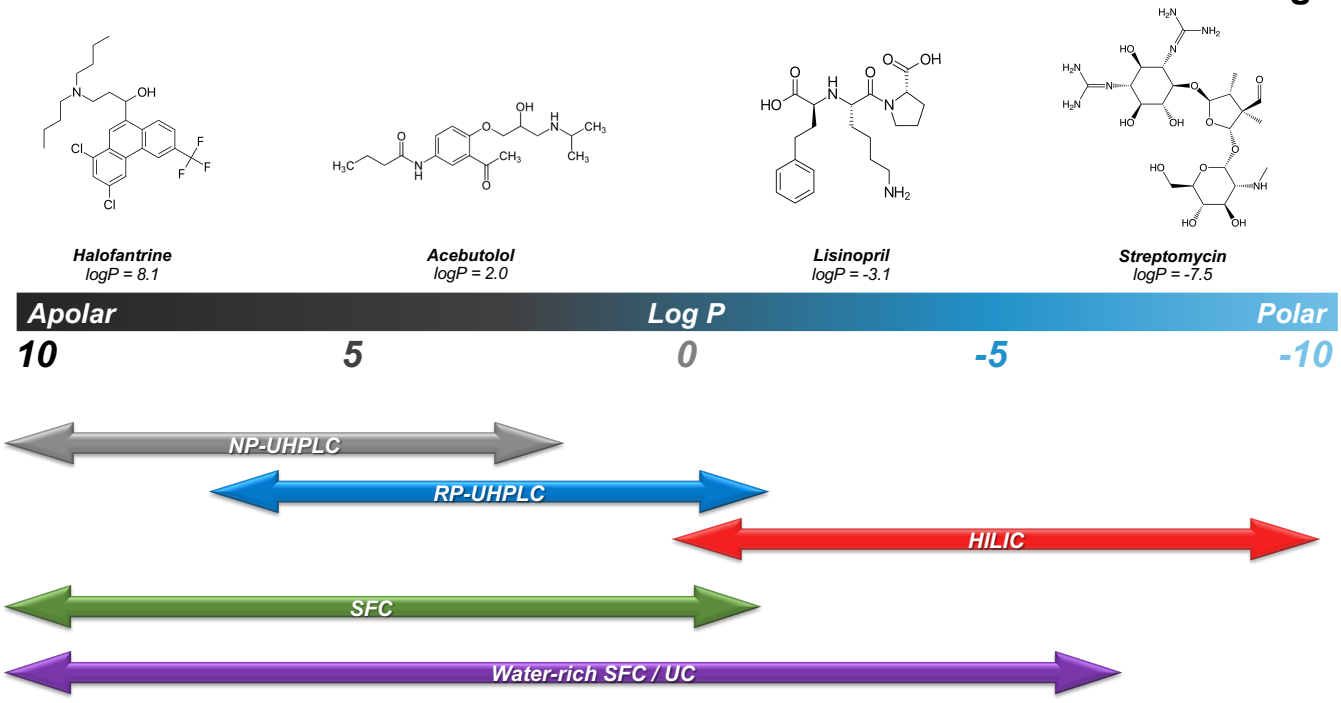


Figure 3

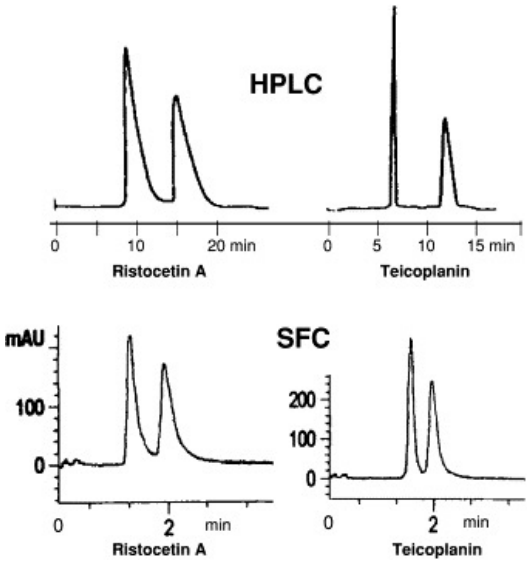


Figure 4

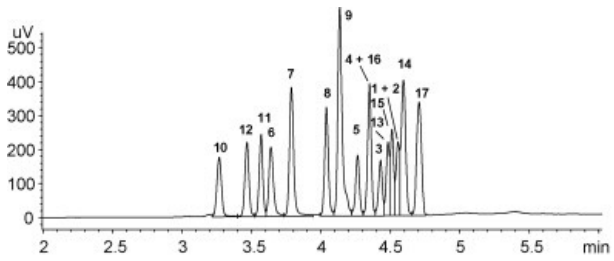


Figure 5

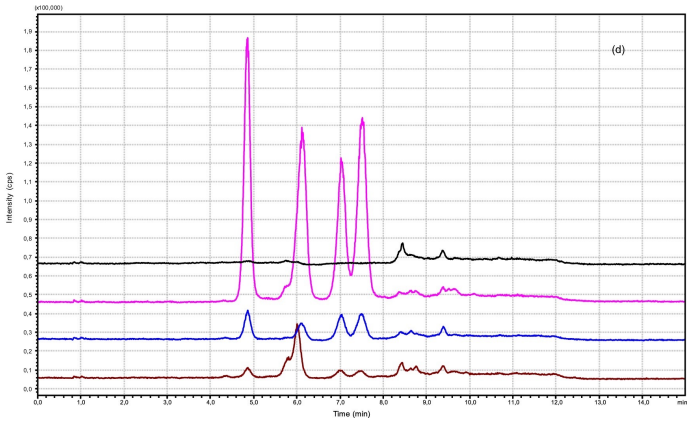
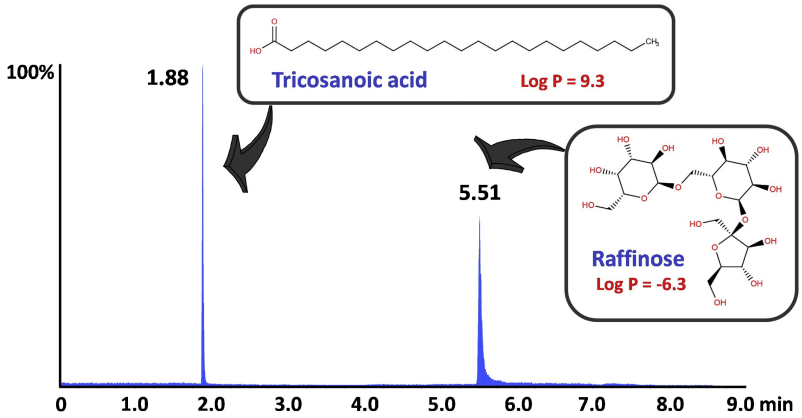


Figure 6



IV.4 Interlaboratory study of a SFC method for the determination of pharmaceutical impurities: evaluation of multi-systems reproducibility

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Interlaboratory study of a supercritical fluid chromatography method for the determination of pharmaceutical impurities: Evaluation of multi-systems reproducibility

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ABSTRACT

Modern supercritical fluid chromatography (SFC) is now a well-established technique, especially in the field of pharmaceutical analysis. We recently demonstrated the transferability and the reproducibility of a SFC-UV method for pharmaceutical impurities by means of an inter-laboratory study. However, as this study involved only one brand of SFC instrumentation (Waters®), the present study extends the purpose to multi-instrumentation evaluation. Specifically, three instrument types, namely Agilent®, Shimadzu®,

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and Waters®, were included through 21 laboratories (n = 7 for each instrument). First, method transfer was performed to assess the separation quality and to set up the specific instrument parameters of Agilent® and Shimadzu® instruments. Second, the inter-laboratory study was performed following a protocol defined by the sending lab. Analytical results were examined regarding consistencies within- and between-laboratories criteria. Afterwards, the method reproducibility was estimated taking into account variances in replicates, between-days and between-laboratories. Reproducibility variance was larger than that observed during the first study involving only one single type of instrumentation. Indeed, we clearly observed an 'instrument type' effect. Moreover, the reproducibility variance was larger when considering all instruments than each type separately which can be attributed to the variability induced by the instrument configuration. Nevertheless, repeatability and reproducibility variances were found to be similar than those described for LC methods; *i.e.* reproducibility as %RSD was around 15 %. These results highlighted the robustness and the power of modern analytical SFC technologies to deliver accurate results for pharmaceutical quality control analysis.

1. Introduction

Modern SFC is now a well-implemented technique in R&D laboratories for a large domain of applications thanks to its versatility [1,2]. The introduction of new analytical SFC instrumentation opened the door to numerous pharmaceutical analysis applications and challenges [3]. Despite these technological innovations and the relevant advantages of this technique, the implementation of new analytical tools in regulated laboratories is still considered as a risk, especially across pharmaceutical quality control laboratories.

Recent publications highlighted the performance of SFC for quantitative analysis with ICH Q2 compliant method validation [4], as well as a framework for impurity fate and purge studies to establish an effective impurity control strategy in the approval of the commercial filing application of new medicines [5]. Furthermore, method transfer and precision evaluation using several instruments was also performed [6,7]. To evaluate properly method reproducibility, the between-laboratories variance should be estimated by means of an inter-laboratory study [8]. The demonstration of method reproducibility is mandatory when deploying a QC method in several laboratories for drug substance or drug product release. Several publications proposed inter-laboratory design and setups, generally focused on LC [9,10]. A few years ago, we proposed the first published inter-laboratory study dedicated to SFC [11]. This study involved salbutamol sulfate impurities determination by means of a previously developed robust SFC-UV method [12]. This collaborative study was accomplished with Waters® UPC^2 instruments in all laboratories. Replicate, day and laboratory variances were considered to estimate the method reproducibility. For this SFC method, repeatability and reproducibility variances were similar or better than those described for LC methods [10].

The objective of the present study was to evaluate SFC reproducibility from several commercially available analytical SFC instruments that are widespread across both academic and industrial sectors, especially in QC laboratories. Using different instrument configurations/designs is more challenging in SFC than in LC due to the specific requirements of this technique regarding the accurate delivery of a compressible mobile phase, the regulation of backpressure, and the injection mode to properly inject a liquid sample within a pressurized chromatographic system. Consequently, method testing and transfer to other instruments was performed in provider laboratory (demo laboratory) before the beginning of the inter-laboratory study. This step was required to establish a study protocol compatible with the three selected SFC instruments from Waters®, Agilent®, and Shimadzu®. Afterwards, the study design was defined, and laboratories were invited to have a balanced design meaning that a similar number of each instrument type was expected. It is often advised to have at least 8 participating laboratories [8]. In the present study, the objective was to maximize the number of laboratories in order to have enough data for each instrument type. In an interlaboratory study, the laboratory terminology defines a set of instrument, operator, and test site. It means that one laboratory can provide several 'laboratories' if they can include several operators with independent equipment at a test site to enable a rigorous study.

The study organization was similar to the previous one, in order to fulfill ISO standards [8]. In each laboratory, the first step consisted of a method test to verify various criteria, namely method selectivity, sensitivity, and system repeatability. This step helped to ensure proper instrument handling and method set-up before the quantitative analysis stage. After the approval of the study coordinator, the content of a target impurity (impurity D) was determined in three independent salbutamol sulfate samples (see supplementary data Figure S1). Indeed, impurity D was selected as a model compound for these inter-laboratory studies as the quantitative performance of the method was similar for all impurities [12]. These samples at different concentration levels of impurity D aimed at covering the validated dosing range. Herein is highlighted the main steps and results of this inter-laboratory study using multi-SFC-systems.

2. Material and methods

2.1. Chemicals and reagents

Salbutamol hemisulfate (> 98.0 %) was purchased from TCI Europe (Zwijndrecht, Belgium) and used as salbutamol hemisulfate standard. Related impurities B, D, F, G and I were provided by EDQM (Strasbourg, France). Salbutamol raw material was split in three batches and each batch was spiked with different amounts of related impurity D to get three salbutamol samples covering the validated dosing range. Each laboratory provided the required solvents and reagents, following minimal quality requirements: methanol, gradient grade; acetonitrile, gradient grade; 2-propanol, analytical grade; water, ULC-MS/SFC grade; ammonium hydroxide, 25 or 28 % w/w, analytical grade; carbon dioxide, 99.995 % purity. Ammonium hydroxide was provided by Merck (Darmstadt, Germany), Sigma-Aldrich (Saint-Louis, USA), Alfa Aesar (Haverhill, USA), Honeywell (Charlotte, USA), VWR (Radnor, USA) and Fujifilm Wako Pure Chemicals (Osaka, Japan). Methanol and 2-propanol were provided by Merck (Darmstadt, Germany), Fisher Scientific (Waltham, USA), Fisher Chemicals (Waltham, USA), VWR (Radnor, USA), EMD (Burlington, USA), J.T. Baker (Phillipsburg, USA), Honeywell (Charlotte, USA) and Fujifilm Wako Pure Chemicals (Osaka, Japan). CO_2 was provided by Air Liquide (Paris, France), PanGas (Delemont, Switzerland), Airgas (Radnor, USA), Rivoira

Gas (Milano, Italy), PraxAir (Danbury, USA), Messer (Bad Soden, Germany) and Iwatani (Houston, USA). Acetonitrile was provided by Merck (Darmstadt, Germany), Fisher Scientific (Waltham, USA), Carlo Erba Reagents (Val de Reuil, France), Sigma-Aldrich (Saint-Louis, USA), J.T. Baker (Phillipsburg, USA), VWR (Radnor, USA), Honeywell (Charlotte, USA) and Fujifilm Wako Pure Chemicals (Osaka, Japan). Water daily produced by Milli-Q dispenser was used in several laboratories. For the others, LC–MS grade water was provided by Fisher Scientific (Waltham, USA), VWR (Radnor, USA), Biosolve (Dieuze, France), Honeywell (Charlotte, USA) and Fujifilm Wako Pure Chemicals (Osaka, Japan).

2.2. Instrumentation

For all instrument types, if applicable, MS or other detectors hyphenated to the chromatographic system were disconnected prior to the experiments. This section describes the configuration of each type of instrument. It also specifically mentions the parameters that have to be set on each system.

2.2.1. Agilent 1260 Infinity II™ SFC system

Each laboratory used a 1260 Infinity II™ system (1260/1290 series) with a variable wavelength detector including a 10 mm path length high-pressure flow cell. The detection was performed at 220 nm with an acquisition frequency of 20 Hz. The injection mode was feed injection (injection volume 2 μL, feed speed 100 μL/min, overfeed volume 4 μL) with 2-propanol as feed solvent. Pre- and post-column temperatures were set at 55 °C and 38 °C, respectively. The temperature of the back pressure regulator was set at 60 °C.

2.2.2. Shimadzu Nexera UC™

Each laboratory used the Nexera UC™ system with a PDA detector working at 220 nm with correction at a reference wavelength of 360 nm with 50 nm bandwidth. The detection cell temperature was set at 40 °C. The detection was performed using an acquisition frequency of 40 Hz and a time constant of 0.32 s. The injection volume was set at 3 μL with a maximal injection loop volume of 20 μL. Water/methanol (50/50, v/v) (700 μL) and 2-propanol (700 μL) were used to wash the sample loop while the needle was washed with methanol. Co-injection with n-heptane was used in some laboratories.

2.2.3. Waters Acquity UPC² ™

Each laboratory used the system with a PDA detector (Waters, Milford, MA, USA). The injector was equipped with a 5 or 10 μL loop operating to inject 2 μL in the partial loop with needle overfill mode. 2-propanol (900 μL) and water/methanol (50/50, v/v) (500 μL) were used as weak and strong needle wash solvents, respectively. Chromatograms were recorded at 220 nm in compensated mode (310–410 nm) with an acquisition frequency of 20 Hz, a resolution of 1.2 nm and a filter time constant of 0.5 s.

2.3. Chromatographic conditions

SFC conditions were reported in a previous publication [12]. Separation conditions were optimized using the Waters system. The UPC² Torus Diethylamine (DEA) 100 × 3.0 mm (particle size of 1.7 μm) analytical column was used at a temperature of 55 °C. One dedicated column was provided to each laboratory, and the whole study was performed using 10 columns. New columns were used and a maximal number of injections performed on each column was defined according to our previous knowledge. The columns were randomly sent to the laboratories. The experiments were executed at a flow rate of 1.5 mL/min and 0.1 % v/v ammonium hydroxide in methanol was used as modifier. The gradient mode was applied, with an initial modifier fraction of 2%, followed by a linear increase to 35 % in 6.5 min. In post-run, the initial mobile phase conditions were reached within 0.5 min followed by 3 min of re-equilibration (total run time 10 min). The backpressure regulator was set at 135 bar (1958 psi). The autosampler temperature was set to 6 °C. The injection volume was adapted to each instrument as detailed above.

2.4. Sample preparation

All solutions were prepared in water/acetonitrile 20/80 v/v. After preparation, all solutions were stored in the dark at 5 °C (±3 °C).

2.4.1. Preliminary testing

Stock solution containing impurities was prepared by transferring accurately weighed amounts of 5 mg impurity B, 5 mg impurity D, 5 mg impurity F and 5 mg impurity G in a volumetric flask of 50.0 mL. Intermediate solution was prepared by weighing an accurate amount of 20 mg salbutamol sulfate and adding 600 μL stock solution in a volumetric flask of 10.0 mL. Then the content of one vial of impurity I was dissolved with 1.0 mL of intermediate solution. This latter solution containing salbutamol sulfate and all related impurities was used to perform the preliminary test (system suitability test (SST) solution).

2.4.2. Inter-laboratory study

Stock solution of impurity D was prepared by adding an accurately weighed amount of 5 mg of impurity D in a volumetric flask of 5.0 mL. Then, calibration standards at 4, 6 and 8 μg mL^{-1} were prepared by means of dilutions (40, 60 and 80 μL of stock solution, respectively, in a 10-mL volumetric flask). Similarly, a QC solution at a concentration of 6 μg mL^{-1} was prepared by means of an independent dilution of the stock solution of impurity D.

Each lab received three salbutamol samples labelled sample A, B and C. These each contained different amounts of impurity D to evaluate the validated dosing range during this study: 0.2 % of impurity D in salbutamol sulfate (sample A), 0.3 % of impurity D (sample C) and 0.4 % of impurity D (sample B). The study was performed in a blind way as the laboratories did not know samples concentration and level. Sample solution was prepared by adding an accurately weighed amount of 20 mg salbutamol sulfate unknown solid sample in a 10-mL volumetric flask. Three independent solutions were prepared for each sample and this protocol was repeated on three days.

2.5. Preliminary testing

The first step of the present study was to perform a training experiment. These first experiments aimed also to verify the reliability of all SFC instruments to provide consistent results. This preliminary testing was implemented to verify several performance criteria: selectivity, retention time stability, peak area variability, and sensitivity. To verify method selectivity and sensitivity, the SST solution was injected 6 times using the above described SFC method. The RSD values should be < 1% for retention times (for all compounds). The RT repeatability evaluation aimed to verify the ability of the SFC instrument to provide repeatable chromatographic profile. The variability of the peak area was automatically considered as valid when RSD values were lower than 3 %. Other values were closely evaluated by the study coordinator, as discussed in the results sections below. Finally, the signal-to-noise ratio (S/N) was calculated according to European Pharmacopoeia requirements by the study coordinator based on an exported chromatogram of SST (signal) and a blank (noise). The objective was to have a unique S/N estimation protocol for all laboratories. The S/N ratio should be higher than 15.

Table 1
Analysis of variance components (p = number of laboratories, c = number of days per laboratory, g = number of replicates per day).

Sources of variability	Mean squares	Estimated variance
Laboratories	$MS_{laboratories} = \frac{cg\Sigma(\bar{x}_i - \bar{x})^2}{p-1}$	$s^2_{laboratories} = \frac{MS_{laboratories} - MS_{days}}{cg}$
Days	$MS_{days} = \frac{g\Sigma\Sigma(\bar{x}_{ij} - \bar{x}_i)^2}{p(c-1)}$	$s^2_{days} = \frac{MS_{days} - MS_{replicates}}{g}$
Replicates	$MS_{replicates} = \frac{\Sigma\Sigma\Sigma(\overline{X}_{ijk} - \overline{X}_{ij})^2}{pc(g-1)}$	$s^2_{replicates} = MS_{replicates}$

2.6. Set-up of the inter-laboratory study

The study involved 21 participating analytical laboratories (p = 21): 10 academic (universities), 4 demonstration laboratories at analytical instrument companies, 6 pharmaceutical companies and 1 research center. The laboratories are coded from 1 to 24 because 3 laboratories canceled their participation after samples labeling and sending. A laboratory means a set of instrument, operator, and test site (reagents, column, *etc.*) [7]. Each laboratory performed the analyses on three different days (series) (c = 3). Per day, the samples were prepared independently in triplicate (g = 3) considering 3 concentrations levels (q = 3) by means of samples A, B and C. A graphical presentation of the study layout per concentration (sample) was proposed in a previous publication [12].

The study layout provides information on three sources of variability, *i.e.* replicates, days, laboratories, which are the main components of method reproducibility. This design enabled determination of repeatability, intermediate precision, and reproducibility during the inter-laboratory study. Each laboratory reported raw data in a validated and locked Excel file. The study coordinator performed all data and statistical analyses using Excel (Microsoft Excel® for Mac) followed by a report verification by the study supervisors.

2.7. Statistical analysis

2.7.1. Scrutiny of results for consistency and outliers

First, the results were critically examined for outliers and stragglers regarding within-laboratory and between-laboratory consistency. This examination was performed using graphical consistency techniques and numerical outlier tests specified in the ISO standards [8]. Tables with critical values for all mentioned tests can be found in the ISO standards [8]. Explanations about the statistical strategy can be found in a previous study [12].

Mandel's *k* values were plotted to graphically evaluate the within-laboratory variability. The indicator values at 1% and 5% significance levels were drawn on the Mandel's plots. For the numerical Cochran's test, the variance is considered to be an outlier when C is larger than the 1% critical value and a straggler when C is smaller than the 1% critical value, but larger than the 5% one. Outliers were noted as ** and stragglers as * in the results tables.

Mandel's *h* values were plotted to graphically evaluate the between-laboratory variation. The Grubb's tests were finally used as a numerical outlier test. For the single Grubb's test performed in the present study, outliers and stragglers gives rise to values exceeding the 1% and 5% critical values, respectively.

2.7.2. Variances estimation

After testing and discarding the outliers, the mean squares between laboratories ($MS_{laboratories}$), between days (MS_{days}) and between replicates ($MS_{replicates}$) were calculated applying the variance analysis detailed in Table 1.

From the mean squares, the repeatability (s^2_r), between-laboratories ($s^2_{laboratories}$) and reproducibility variances (s^2_R) were estimated [10]. In the present study, the protocol layout involved three independent series for each laboratory by means of three different days. Consequently, reproducibility was estimated according to [10]:

$$s^2_r = s^2_{replicates} \quad (1)$$

$$s^2_R = s^2_{replicates} + s^2_{days} + s^2_{laboratories} \quad (2)$$

Moreover, intermediate precision can also be estimated using the replicate and day variances

2.7.3. Uncertainty estimation

The reproducibility variance allowed the estimation of the standard uncertainty u_x using the following equation:

$$u_x = \sqrt{s^2_R} \quad (3)$$

Therefore, the expanded uncertainty U_x could be calculated as:

$$U_x = 2u_x \quad (4)$$

using a coverage factor k = 2 [13].

3. Results and discussion

3.1. Method transfer

To begin, the separation was performed on both Shimadzu® and Agilent® instrument using PDA detector at their respective demo labs. The retention profile was compared with a reference chromatogram obtained on the same column. Testing was performed using salbutamol sulfate spiked with all impurities at a concentration corresponding to the tolerated amount. Retention times between systems were comparable and elution profiles were similar. Pressure observed at the beginning of the gradient and pressure slope during the gradient were also comparable (±10 bar due to equipment configuration and increase of 70 bar on both systems). The variability of retention times was also recorded, and RSD values were always lower than 0.1 % for Agilent® and Shimadzu® instruments, except for the first eluted peak using the Shimadzu® instrument (0.33 %). These values were similar to those of the reference method (*i.e.*, lower than 0.1 %), except for the first eluted compound using Shimadzu system was slightly higher, probably due to the higher compressibility of the fluid at the beginning of the gradient. Nevertheless, this variability was only minor and within the acceptance criteria. Peak area repeatability was also checked, with acceptable RSD values. Despite these suitable results, both systems were not compliant to our requirements using the method highlighted in section 2.3 regarding the sensitivity for impurity D at the target concentration (0.3 % m/m), *i.e.* S/N of 3 and 12 for Agilent® and Shimadzu® instruments, respectively. For Shimadzu® instrument, the lower sensitivity was partly explained by the path length of the UV detector cell, *i.e.* 6 mm instead of 10 mm. The injection volume was increased (3, 4 and 5 μL) to minimize the detector cell effect. Unfortunately, peak splitting of the two or three first eluted peaks was observed due to the injection of a high volume of MeOH/water 80/20 v/v into the supercritical mobile phase. Some additional testing regarding solubility and peak shape were performed to propose a suitable injection solvent. The use of acetonitrile instead of methanol enabled to avoid peak splitting with an injection volume of 3 μL balancing the shorter optic path length of Shimadzu® detector. The insufficient sensitivity of the Agilent setup could be explained by an elevated baseline noise / drift and an overall lower peak intensity. To overcome this issue, additional tests were performed using UV variable wavelength detector (VWD) instead of PDA. This detector is indeed more sensitive and specifically designed for low flow rates at elevated pressures. The use of this detector helped reaching a suitable

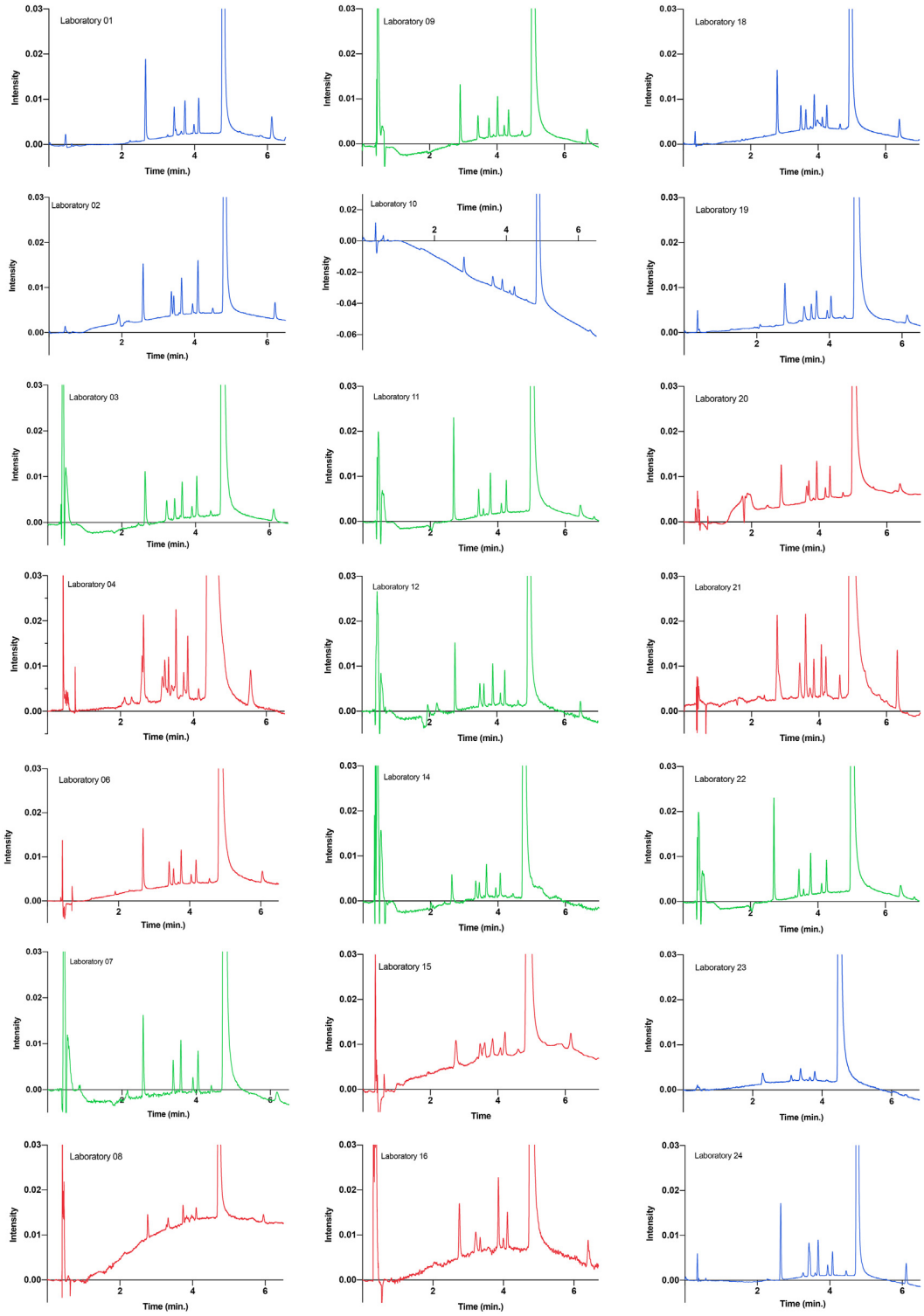

Fig. 1. Chromatogram obtained in each laboratory during the familiarization step. Blue Waters instrument – Green Agilent instrument – Red Shimadzu instrument.

method sensitivity, and is the recommended setup for applications using flow-rates below 2.5 mL/min. The VWD is not the most widespread in Agilent® SFC configurations used in the laboratories involved in the present study. In this context, Agilent® installed a loan VWD detector available in each laboratory involved in the study. To summarize, this part focusing on method transfer helped to set up the instruments and method settings, to propose a study protocol compatible with the three instrument types considered in the present study.

3.2. Familiarization step

In each participating lab, the first step was to check their ability to perform the salbutamol sulfate impurities analysis. The chro-

Table 2
Results of performance preliminary testing (ND = not detected).

Lab	Instrument	Retention times (min) and RSD (%)						Peak area RSD (%, n = 6)				
		D	G	I	B	API	F	D	G	I	B	F
01	Waters	2.63 (0.03)	3.34 (0.03)	3.58 (0.02)	3.93 (0.01)	4.76 (0.04)	5.77 (0.01)	0.67	2.17	0.23	1.16	0.93
02	Waters	2.59 (0.04)	3.37 (0.04)	3.65 (0.02)	4.10 (0.01)	4.83 (0.01)	6.22 (0.02)	0.84	0.40	1.88	1.41	1.18
03	Agilent	2.63 (0.15)	3.22 (0.10)	3.64 (0.10)	4.03 (0.10)	4.72 (0.08)	6.11 (0.22)	2.82	10.32	2.59	2.65	19.44
04	Shimadzu	2.62 (0.30)	3.21 (0.08)	3.56 (0.26)	3.86 (0.39)	4.45 (0.37)	5.62 (0.81)	0.81	0.86	1.90	1.76	1.90
06	Shimadzu	2.69 (0.20)	3.43 (0.16)	3.77 (0.11)	4.18 (0.14)	4.86 (0.15)	6.05 (0.29)	1.73	1.87	1.03	1.79	1.31
07	Agilent	2.57 (0.03)	3.38 (0.02)	3.59 (0.02)	4.05 (0.02)	4.76 (0.02)	6.18 (0.08)	2.14	1.42	3.60	2.39	2.57
08	Shimadzu	2.74 (0.49)	3.33 (0.11)	3.72 (0.49)	4.07 (0.51)	4.69 (0.54)	5.89 (0.79)	4.73	5.76	6.55	5.25	5.21
09	Agilent	2.91 (0.03)	3.43 (0.04)	4.01 (0.03)	4.34 (0.02)	5.06 (0.02)	6.67 (0.02)	1.77	1.48	1.84	1.05	1.27
10	Waters	2.84 (<0.005)	3.64 (0.11)	3.91 (0.10)	4.24 (0.10)	4.90 (<0.005)	6.35 (0.06)	3.17	2.96	2.56	1.82	10.80
11	Agilent	2.70 (0.02)	3.44 (0.02)	3.78 (0.02)	4.26 (0.02)	5.01 (0.03)	6.46 (0.02)	0.88	0.94	0.78	1.15	2.09
12	Agilent	2.76 (0.04)	3.49 (0.04)	3.88 (0.01)	4.23 (0.02)	4.94 (<0.005)	6.48 (0.06)	0.83	1.93	1.76	1.74	1.99
14	Agilent	2.64 (0.03)	3.35 (0.08)	3.66 (0.02)	4.07 (0.04)	4.77 (0.04)	ND	6.79	7.26	4.56	9.04	ND
15	Shimadzu	2.74 (0.73)	3.46 (0.40)	3.83 (0.55)	4.20 (0.45)	4.89 (0.33)	6.15 (0.36)	1.96	1.72	1.85	1.59	1.55
16	Shimadzu	2.74 (0.17)	3.21 (0.19)	3.86 (0.16)	4.12 (0.20)	4.78 (0.22)	6.43 (0.31)	1.87	1.97	1.91	1.46	1.84
18	Waters	2.79 (<0.005)	3.49 (<0.005)	3.89 (0.10)	4.26 (<0.005)	4.96 (0.10)	6.41 (<0.005)	0.44	1.05	0.43	0.64	1.38
19	Waters	2.78 (0.09)	3.31 (0.07)	3.65 (0.05)	4.05 (0.04)	4.73 (0.04)	6.14 (0.04)	1.32	1.50	0.59	1.65	1.95
20	Shimadzu	2.85 (0.50)	3.61 (0.29)	3.90 (0.23)	4.16 (0.21)	5.00 (0.15)	6.37 (0.13)	0.89	1.54	1.14	1.09	1.44
21	Shimadzu	2.76 (0.10)	3.60 (0.05)	3.85 (0.06)	4.07 (0.06)	4.97 (0.04)	6.33 (0.05)	1.67	1.07	1.74	1.91	1.23
22	Agilent	2.62 (0.81)	3.39 (1.03)	3.69 (1.00)	4.13 (0.92)	4.88 (0.02)	6.27 (0.02)	0.81	1.03	1.00	0.92	1.33
23	Waters	2.31 (0.03)	3.13 (0.02)	3.40 (0.02)	3.80 (0.01)	4.51 (0.02)	6.08 (0.13)	2.33	6.13	4.82	3.45	16.02
24	Waters	2.65 (0.02)	3.42 (0.04)	3.68 (0.02)	4.07 (0.03)	4.76 (0.02)	6.11 (0.02)	1.19	0.59	1.18	0.83	2.64
Mean		2.69 (4.71)	3.39 (3.97)	3.74 (3.98)	4.11 (3.25)	4.82 (3.22)	6.20 (4.07)	1.69	2.57	2.09	2.13	3.54

matograms obtained in each laboratory are displayed in Fig. 1 and supplementary data Figure S2. As illustrated in this figure, the method was successfully transferred to all laboratories regarding the separation profile (retention and peak widths). However, some differences of selectivity and sensitivity were observed. Regarding the selectivity, most of the individual laboratories reported an adequate separation of API and related impurities. The separation reported in laboratories 4, 8, and 15 were not optimal in comparison to other labs, and a lack of selectivity was observed for impurities eluted between impurity D (first peak) and the API. These laboratories reported a relatively low pressure at the beginning of the gradient (see supplementary data Table 1), with a conventional pressure increase along the gradient (approximately 70 bar). This low pressure could not explain fully this lack of selectivity, as other labs achieved suitable peaks separations with similar pressure values.

Table 2 presents the results of the preliminary performance test. The observed retention time variability with six replicates was no more than 1.0 %. This variability was instrument-dependent because RSD values lower than 0.1 % were obtained for more than half of participating laboratories. In general, the retention times repeatability was worse for Shimadzu® instruments. Nevertheless, the overall retention time repeatability was fully acceptable with RSD values lower than 5% considering all the 21 laboratories. Finally, the peak area variability was assessed by performing six consecutive injections. Except for laboratories 3, 14 and 23, all laboratories reached a suitable peak area variability, lower than 3%. Moreover, in laboratory 14, impurity F was not detected (or

Table 3
Summary of the preliminary test for each type of SFC instrument.

System	Mean retention times (min) and RSD (%)						Mean peak area RSD (%, n = 6)				
	D	G	I	B	API	F	D	G	I	B	F
Agilent	2.69 (4.26)	3.39 (2.55)	3.75 (4.00)	4.15 (2.87)	4.86 (2.71)	6.36 (3.32)	2.29	3.48	2.30	2.71	4.78
Shimadzu	2.73 (2.55)	3.41 (4.87)	3.78 (3.05)	4.09 (2.81)	4.81 (3.94)	6.12 (4.77)	1.95	2.11	2.30	2.12	2.07
Waters	2.66 (6.74)	3.39 (4.67)	3.68 (4.80)	4.06 (3.99)	4.78 (3.03)	6.15 (3.40)	1.42	2.11	1.67	1.57	4.01

Table 4
Sensitivity – S/N.

Lab	Instrument	Signal/noise ratio				
		D	G	I	B	F
01	Waters	50	30	36	34	20
02	Waters	46	94	116	140	28
03	Agilent	38	14	10	48	10
04	Shimadzu	62	44	60	50	18
06	Shimadzu	72	30	66	66	38
07	Agilent	14	18	26	22	32
08	Shimadzu	14	12	40	8	32
09	Agilent	32	16	12	16	18
10	Waters	20	52	10	8	54
11	Agilent	106	22	40	92	28
12	Agilent	40	18	34	32	3
14	Agilent	26	10	22	34	ND
15	Shimadzu	14	14	18	58	36
16	Shimadzu	40	28	44	64	6
18	Waters	58	40	12	18	24
19	Waters	44	18	48	114	16
20	Shimadzu	52	30	102	70	32
21	Shimadzu	104	80	56	56	20
22	Agilent	88	22	56	64	12
23	Waters	16	20	34	32	3
24	Waters	50	40	42	52	80
Mean ± sd		23 ± 14	16 ± 11	21 ± 14	26 ± 17	13 ± 9

* ND: not detected.

eluted) despite performing several additional tests and complete instrument maintenance. As the variability of impurity D peak area (target compound of the present study) was lower than 3.0 % for all laboratories (without lab 14), they were selected for further investigations.

These data were also computed considering each SFC instrument independently (Table 3). We observed similar retention time trend between the three instruments, with a larger retention time variability for the first eluted peaks on Waters® systems. In general, the peak area variability was slightly better for this SFC instrument. Finally, the results were also evaluated in terms of sensitivity by means of signal-to-noise ratio estimation, as illustrated in Table 4. The variability in the S/N values observed could be easily explained by the amount of noise and/or the signal intensity reached in each laboratory. The chromatograms in Fig. 1 highlighted quite well the different S/N measured. Some laboratories did not meet the specification for impurity D sensitivity, *i.e.* S/N not lower than 15, with a value just below the limit, *i.e.* S/N of 14 for labs 7 and 8., Because of this limit value and of their suitable peak area variability for this impurity, they were still considered for the next step of the study. In conclusion, all laboratories performed the reproducibility estimation step, except laboratory 14.

3.3. Inter-laboratory study – quantitative results

Using a validated and locked Excel sheet, each laboratory reported the mass content (% m/m) of impurity D in salbutamol sulfate A, B and C samples. Before the evaluation of the sample results, data analysis was performed regarding three criteria: injection repeatability of the standard solutions, QC solution recovery, and calibration curve linearity. Laboratory 15 provided RSD values from 4 to 48 % for three injections. Moreover, for series 1, a recovery of 130 % was reported for the QC solution. Finally, determination coefficient (R^2) of calibration curve was estimated at 0.86 and 0.97 for series 1 and 2, respectively. Only the third series seemed reliable, which points at an operator error, rather than an instrument issue. The injection variability also highlighted a system issue, which was not detected during the preliminary step. This laboratory was then discarded because the data were not reliable. Laboratory 23 reported RSD values from 6 to 30 % for three injections. Moreover, recoveries between 80 and 90 % were reported for the QC solution. This laboratory was also discarded, because the data were not reliable. The results of the 18 remaining laboratories are summarized in supplementary data Table S2 and supplementary Figure S3. To avoid such instrument related issues (injection variability), the laboratories that were waiting to perform the study were asked to perform an instrument performance qualification (at least on the injector module) before starting the study. The use of qualified instrumentations is a keystone of analytical methods to guarantee the quality of the results [14]. To overcome this unexpected issue, the qualification of the instrument was implemented during the study. Moreover, the use of multi-configurations instruments (*i.e.*, SFE-SFC hyphenation or direct injection SFC) could lead to injection issues. The implementation of qualification helped to identify them before the beginning of quantitative measurements. Unfortunately, instrument qualification and intensive analyst training is not well implemented in some R&D laboratories. These two aspects are mandatory to perform QC analysis and are of prime importance for the implementation of new technologies such as SFC.

3.3.1. Scrutiny of results for consistency and outliers

Within- and between-laboratory consistencies were examined by means of the graphical Mandel's methods and with numerical outlier tests.

3.3.1.1. Within-laboratory variance tests. Mandel's *k* plotting and Cochran's test were used to verify if the within-laboratory variance of some laboratories was not considerably larger than in other participating laboratories. These tests were performed considering nine measurements (n = 9 by means of three replicates on three series) for each sample in each lab. Results are shown in Table 5 and Fig. 2. On the Mandel'*k* plot, indicator lines at 1 and 5% significance

Table 5
Within-laboratory results consistency (**outlier, *straggler).

Mandel's *k* statistics			
	Sample A	Sample B	Sample C
Lab 01	0.9515	0.1587	0.5404
Lab 02	0.7663	0.7754	0.6156
Lab 03	0.9746	0.8751	0.8022
Lab 04	0.5516	0.4910	0.4861
Lab 06	1.0234	1.0249	0.7431
Lab 07	0.8472	0.1072	0.2382
Lab 08	0.7982	0.4373	0.8975
Lab 09	0.4480	0.4640	0.5374
Lab 10	1.5737**	2.4018**	2.3962**
Lab 11	0.7128	0.2231	0.2671
Lab 12	0.2769	0.2791	0.3368
Lab 16	0.1817	0.2770	0.3445
Lab 18	0.2304	0.3191	0.3376
Lab 19	0.4176	0.2283	0.3751
Lab 20	0.1954	0.1410	0.3918
Lab 21	2.7194**	2.4975**	2.7818**
Lab 22	1.3061	1.5511**	0.6935
Lab 24	0.4350	0.3287	0.2708
Indicator values for Mandel's *k* statistics (p = 18, n = 9)			
5 % level		1.38	
1 % level		1.55	
Cochran's test			
	Sample A	Sample B	Sample C
C	0.4108** (outlier lab 21)	0.3465** (outlier lab 21)	0.4299** (outlier lab 21)
Critical values (p = 18, n = 9)			
5 % level		0.1556	
1 % level		0.2046	
Second Cochran's test (after elimination of outliers)			
C	0.2335** (outlier lab 10)	0.4904**(outlier lab 10)	0.5595**(outlier lab 10)
Critical values (p = 17, n = 9)			
5 % level		0.1512	
1 % level		0.1931	
Third Cochran's test (after elimination of outliers)			
C	0.2099** (outlier lab 22)	0.4041**(outlier lab 22)	0.1762*(straggler lab 22)
Critical values (p = 16, n = 9)			
5 % level		0.1467	
1 % level		0.1873	

levels were drawn. Laboratory 21 and 10 tend to show a higher repeatability variance than the other labs, highlighting their worse repeatability. Numerical outlier technique was also performed by means of Cochran's test (see Table 5). This test highlighted outlier values for labs 21 at all concentration levels (samples A, B, and C). A second Cochran's test showed an outlier value for lab 10 at all concentration levels. The third Cochran's test showed outlier and straggler values for lab 22. However, it is important to keep in mind that the repetition of statistical tests may lead to excessive rejection. Moreover, this laboratory presented only one Mandel's *k* outlier value for sample B, with a Mandel's *k* value just above the limit. In this context, labs 21 and 10 were considered as outlying laboratories, while lab 22 was not discarded at this stage.

3.3.1.2. Between laboratories variance tests. Mandel's *h* plotting and Grubbs' test were used to verify if there were laboratories with deviating results compared to those of the other laboratories. Results are shown in Table 6 and Fig. 3. On the Mandel's *h* plot, indicator lines at 1 and 5% significance levels were drawn. As illustrated in Fig. 3, laboratories 3 and 4 tended to report higher concentrations than the other laboratories, but Mandel's *k* values remained between both significance levels. Moreover, for some samples analyzed by these laboratories, Mandel's *k* values were below the 5 % significance level. This plot also highlighted the quite balanced distribution of reported values around the mean value. Grubb's tests were performed on mean value reported by each lab. As illustrated in Table 6, no outlier value was reported. Straggler value were reported for laboratory 3 (sample A) and laboratory 4 (sample B and C), confirming Mandel's *k* results. These Grubb's tests could also be performed on single measurement. Results are also reported in Table 6. Grubb's test highlighted only one straggler value for lab 3 sample A.

3.3.1.3. Results consistency. As defined in ISO standards, results outliers with the numerical technique, or stragglers with the numerical technique and exceeding the 1% critical level on the Mandel's plot, should be removed from the data set. Table 7 summarizes the results and highlights outlier values. As required by ISO standards, outlier laboratories (lab 10 and lab 21) were discarded for method variances estimation. Nevertheless, the outlier result obtained by lab 22 (sample B) was kept in the data for two main reasons: (i) Mandel's *k* value was just at the limit; (ii) to avoid the risk of exceeding results rejection due to subsequent Cochran's tests. The outlier values obtained were mainly explained by sample

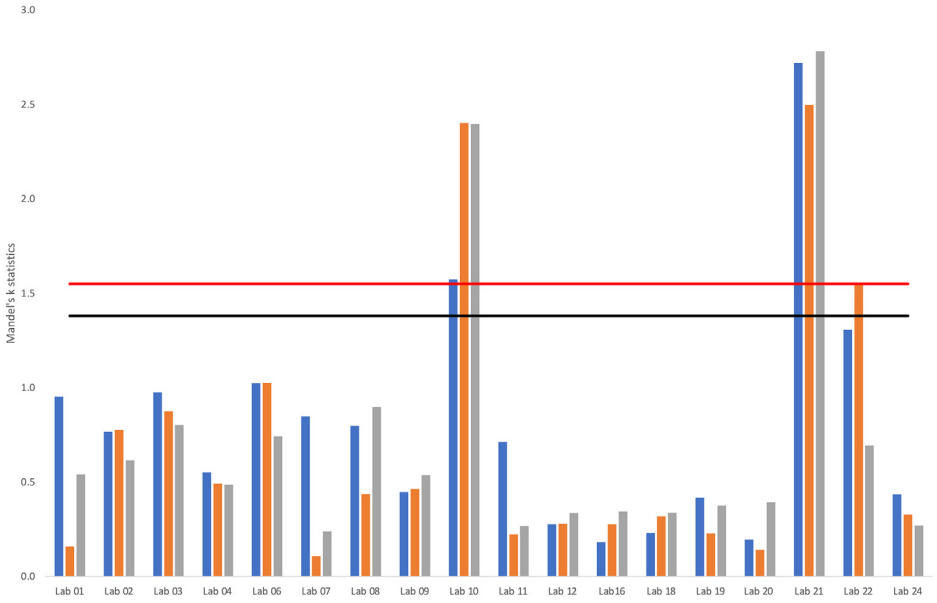

Fig. 2. Mandel's *k* plotting – within-laboratory consistency. Samples A, B and C were represented in blue, orange and grey, respectively. Indicator lines at 1 % (red) and 5 % (black) significance levels.

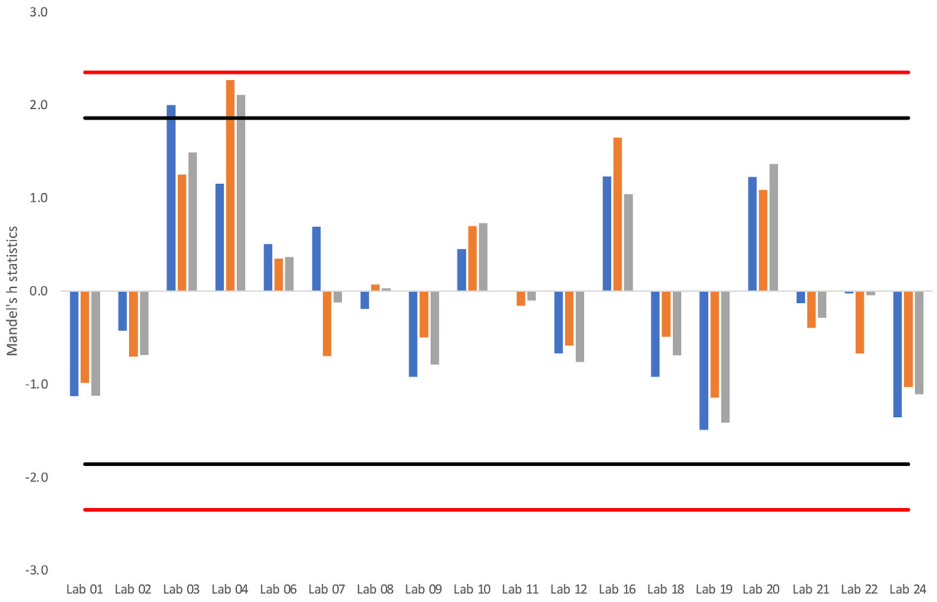

Fig. 3. Mandel's *h* plotting – within-laboratory consistency. Samples A, B and C were represented in blue, orange and grey, respectively. Indicator lines at 1 % (red) and 5 % (black) significance levels.

and standard preparation. This hypothesis was corroborated by the calibration curves obtained in these laboratories. Indeed, the calibration curves were not similar regarding the intensity between days, showing a standard preparation variability. This step of the analytical protocol was similar whatever the analytical technique used for the quantitative analysis and is not related to the specific case of SFC.

3.3.2. *Variance estimation*

The main objective of an interlaboratory study was to estimate method reproducibility. Indeed, this criterion is a part of the method precision which is not estimated during method validation performed using one instrument in one laboratory [8]. To estimate method reproducibility, a variance analysis was performed as illustrated in Table 8. The first important topic is that the "laboratory" factor is the major contributor to method variance with a proportion close to 85–90% for all concentration levels. This part of method variability is higher than the one observed in previous study, which was around 70 % [12]. Regarding the impact of "replicate" and "day" effect, their contribution was similar with a larger impact of the day factor. To confirm the impact of multi-systems usage to the overall laboratory effect, the present data were analyzed considering each type of SFC system independently, *i.e.* as if three studies were performed, one for each SFC system type. The results are summarized in Table 9. The overall system contribution to reproducibility is estimated around 70 % when the results were analyzed independently for each instrument type. Moreover, the "laboratory" factor effect represents around 53 % of method

Table 6
Between-laboratories results consistency.

Mandel's *h* statistics			
	Sample A	Sample B	Sample C
Lab 01	−1.1308	−0.9864	−1.1236
Lab 02	−0.4240	−0.7042	−0.6883
Lab 03	2.0008*	1.2516	1.4877
Lab 04	1.1522	2.2661*	2.1043*
Lab 06	0.5063	0.3487	0.3669
Lab 07	0.6949	−0.6988	−0.1205
Lab 08	−0.1901	0.0688	0.0314
Lab 09	−0.9222	−0.4995	−0.7880
Lab 10	0.4517	0.6973	0.7312
Lab 11	−0.0038	−0.1567	−0.1031
Lab 12	−0.6698	−0.5828	−0.7599
Lab 16	1.2303	1.6457	1.0409
Lab 18	−0.9186	−0.4913	−0.6899
Lab 19	−1.4908	−1.1468	−1.4125
Lab 20	1.2235	1.0863	1.3640
Lab 21	−0.1308	−0.3969	−0.2870
Lab 22	−0.0232	−0.6698	−0.0451
Lab 24	−1.3556	−1.0315	−1.1087
Indicator values for Mandel's *h* statistics (p = 18)			
5 % level		|2.35|	
1 % level		|1.86|	
Grubb's test on lab mean (one outlying observation)			
	Sample A	Sample B	Sample C
G_p	2.0008	2.2661	2.0433
G_1	1.4908	1.1468	1.3686
Critical values (p = 18)			
Grubb's test on individual measurement (one outlying observation)			
	Sample A	Sample B	Sample C
G_p	2.6995*	2.3608	2.5144
G_1	1.6240	2.6122	1.6697
	Straggler lab3		
Critical values (p = 18)			

Table 7
Summary of labs results.

Average impurity D content in salbutamol sulfate (% m/m)			
	Sample A	Sample B	Sample C
Lab 01	0.2072	0.3856	0.2618
Lab 02	0.2335	0.4017	0.2804
Lab 03	0.3238	0.5139	0.3733
Lab 04	0.2922	0.5721	0.3996
Lab 06	0.2682	0.4621	0.3254
Lab 07	0.2752	0.4021	0.3046
Lab 08	0.2422	0.4461	0.3111
Lab 09	0.2150	0.4135	0.2761
Lab 10	0.2661**	0.4821**	0.3410**
Lab 11	0.2492	0.4331	0.3054
Lab 12	0.2244	0.4087	0.2773
Lab 16	0.2951	0.5365	0.3542
Lab 18	0.2151	0.4139	0.2803
Lab 19	0.1938	0.3764	0.2495
Lab 20	0.2949	0.5044	0.3680
Lab 21	0.2444**	0.4194**	0.2975**
Lab 22	0.2485	0.4037	0.3079
Lab 24	0.1988	0.3830	0.2625
Mean	0.2493	0.4421	0.3098

Table 8
Estimation of the variance components.

Sources of variability	Impurity D at 0.2 % (sample A)	Impurity D at 0.3 % (sample C)	Impurity D at 0.4 % (sample B)
Variances			
Laboratories ($s^2_{laboratories}$)	1.49×10^{-3}	1.94×10^{-3}	3.47×10^{-3}
Days (s^2_{days})	1.66×10^{-4}	1.13×10^{-4}	2.80×10^{-4}
Replicates ($s^2_{replicates}$)	7.25×10^{-5}	5.91×10^{-5}	2.07×10^{-4}
Repeatability variance (s_r^2)	7.25×10^{-5}	5.91×10^{-5}	2.07×10^{-4}
Reproducibility variance (s_R^2)	1.73×10^{-3}	2.12×10^{-3}	3.96×10^{-3}
Ratio $(s_R^2)/(s_r^2)$	23.81	35.81	19.07
Repeatability sd (s_r)	8.51×10^{-3}	7.67×10^{-3}	1.44×10^{-2}
Reproducibility sd (s_R)	4.15×10^{-2}	4.60×10^{-2}	6.29×10^{-2}
Ratio $(s_R)/(s_r)$	4.48	5.98	4.37
Repeatability RSD (%)	3.43 %	2.49 %	3.26 %
Intermediate precision RSD (%)	6.21 %	5.00 %	4.25 %
Reproducibility RSD (%)	16.72 %	14.90 %	14.26 %

Table 9
Estimation of the variance components considering each SFC instrument.

	Impurity D at 0.2 % (sample A)			
	All SFC systems (n = 16)	SFC Waters systems (n = 5)	SFC Agilent systems (n = 6)	SFC Shimadzu systems (n = 5)
Repeatability RSD (%)	3.43 %	2.52 %	4.69 %	2.01 %
Intermediate precision RSD (%)	6.21 %	6.54 %	6.88 %	5.11 %
Reproducibility RSD (%)	16.72 %	9.24 %	16.53 %	9.37 %
Ratio $(s_R)/(s_r)$	4.48	3.65	3.52	4.66
	Impurity D at 0.3 % (sample C)			
	All SFC systems (n = 16)	SFC Waters systems (n = 5)	SFC Agilent systems (n = 6)	SFC Shimadzu systems (n = 5)
Repeatability RSD (%)	2.49 %	2.91 %	2.00 %	2.59 %
Intermediate precision RSD (%)	5.00 %	4.02 %	4.30 %	4.27 %
Reproducibility RSD (%)	14.90 %	6.14 %	12.05 %	10.62 %
Ratio $(s_R)/(s_r)$	5.98	2.10	6.02	4.09
	Impurity D at 0.4 % (sample B)			
	All SFC systems (n = 16)	SFC Waters systems (n = 5)	SFC Agilent systems (n = 6)	SFC Shimadzu systems (n = 5)
Repeatability RSD (%)	3.26 %	3.57 %	4.18 %	1.78 %
Intermediate precision RSD (%)	4.25 %	3.59 %	6.44 %	4.17 %
Reproducibility RSD (%)	14.26 %	5.18 %	11.49 %	10.89 %
Ratio $(s_R)/(s_r)$	4.37	1.45	2.74	6.13

Table 10
Estimation of the overall measurement uncertainty.

	Impurity D at 0.2 % (sample A)	Impurity D at 0.3 % (sample C)	Impurity D at 0.4 % (sample B)
Expanded uncertainty (% m/m)	0.08	0.09	0.13
Relative expanded uncertainty (%)	33.43 %	29.82 %	28.52 %

reproducibility variance for Waters® instrument, while this value is close to 80 % for other systems. Two main messages can be drawn: (i) the use of several instrument types impacts method reproducibility by increasing the laboratory effect; (ii) the laboratory effect is lower for Waters® instruments. In fairness, it is important to mention that the method was developed using a Waters instrument [11] with settings (like flow rate) that could be ideal for this equipment but not for the others. Agilent® and Shimadzu® systems are more versatile because of their modular configuration. It is indeed possible to work in LC or SFC modes with such systems, and SFE-SFC can be directly hyphenated with the Shimadzu® system. In the present study, working with a SFC-dedicated system (Waters®) seems to minimize the laboratory factor thanks to its dedicated configuration and method settings optimized for this system. The minimization of the laboratory effect also helped to reduce reproducibility variance. Indeed, as illustrated in Table 9, the reproducibility obtained using the Waters® SFC instrument is better than other instruments for the whole dosing range.

It is generally expected to have the reproducibility standard deviation 2–4 times higher than the repeatability standard deviation [15]. In the present study, ratios between 4 and 6 were measured between the reproducibility and the repeatability standard deviation. These values highlight the importance of the laboratory effect to the overall method variability. This laboratory contribution to the total variance could be explained by: (i) the use of several SFC systems from different equipment manufacturers; (ii) the recent SFC implementation in some participating laboratories; and (iii) the study design, including R&D laboratories not always familiar with impurities quantification. Moreover, the difficulty to handle low masses and low dilution volumes is also a potential issue, independent of the analytical technique.

In order to present some more intuitive values, relative standard deviations were calculated for both repeatability, intermediate precision, and reproducibility. RSD reproducibility values below 17 % for the first concentration level (LLOQ) and below 15 % for the other concentration levels were obtained in this study. Considering all sources of variability, *i.e.* replicates, days, and 16 laboratories (meaning 16 instruments including three different instrument types), these good RSD values again clearly highlighted the reliability of this SFC method for the quantification of salbutamol sulfate impurity D. The reproducibility RSD were also estimated for each SFC instrument cluster (Table 9). As previously highlighted, using the same instrument in all laboratories helped to improved method reproducibility, mainly by the decrease of the "laboratory" factor. It is also noticeable that the reproducibility is better for Waters® instrument with RSD values from 5 to 10 %, confirming the results obtained during the previous study [12]. As mentioned above, this could be explained by the fact that the method was developed using Waters instrument [11] with settings (like flow rate) that could be ideal for this equipment but not for the others. The number of laboratories for each instrument type is lower than the minimum number of testing labs required to perform an inter-laboratory study [8]. Consequently, these results should be confirmed using a larger panel of labs.

3.3.3. Measurement uncertainty evaluation

The expanded uncertainty values are available in Table 10. These values mean that, for a non-conform sample (salbutamol sulfate containing 0.4 % of impurity D), the result was expected to have an expanded uncertainty of 0.13 % m/m. Therefore, 95 % of the reported values are expected to be comprised between 0.27 and 0.53 %. As illustrated in supplementary Table 2, the individual measurements fulfilled this expectation, since 9 out of 171 measurements in sample A (corresponding to 5%) were outside the expanded-uncertainty range. Using the mean value of each laboratory, only one laboratory was outside the range for concentrations of 0.3 and 0.4 % (samples C and B) and no laboratory was outside the range for the concentration of 0.2 %. The laboratory outside the range was the one previously highlighted with Mandel's *k* values between both significance levels, showing a tend to report higher concentration than the other laboratories (lab 4). As observed for the variance estimation, the relative expanded uncertainty values were also lower or equivalent those described in the literature for LC methods using a similar study protocol and concentration level [9].

4. Conclusion

A collaborative study was carried out using a defined SFC method to determine the content of impurity D in salbutamol sulfate API. After a first successful study involving only one instrumentation brand (Waters®), the present study expanded to different SFC manufacturers' equipment (Waters®, Agilent®, Shimadzu®). The method was first transferred and adapted to face the challenge of different SFC instrument designs and configurations. This step highlighted the impact of instrument type to method transfer, as being more critical than in conventional LC. Afterwards, the method reproducibility was estimated by taking into account replicates, days, and laboratory variances. For this SFC method, in the context of multi-systems use, repeatability and reproducibility variances were similar than those described for LC methods. The variances were also estimated for each group of SFC instrument. This estimation highlighted the impact of multi-systems used in the overall analysis variability. Generally, the variance was lower within an instrument group than the overall variance. Moreover, based on the results, it seemed that the instrument type on which the method has been optimized provided better quantitative performance compared to the systems to which the method has been transferred. This information is important for method transfer between laboratories from the same company of between partner laboratories, since transferability will be streamlined if the same instrument (same manufacturer) is used in both laboratories. To summarize, the reproducibility values highlighted the reliability of the method and its potential use for QC analysis across different laboratories and equipment brands. It is also important to point out that reproducibility evaluation is mandatory to propose the method as a real alternative to normative methods. In the context of normative method, it is also important to demonstrate the usability of several instrument types. For the first time, the quantitative and robust performance of modern SFC was demonstrated by means of a collaborative study involving three instrument types, *i.e.* Agilent®, Shimadzu® and Waters®, illustrating the power of SFC for pharmaceutical quality control.

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Takeshi Bamba: Supervision, Writing – review & editing
Florent Rouvière: Investigation
Sabine Heinisch: Supervision
Davy Guillarme: Conceptualization, Supervision, Writing – review & editing, Project administration
Philippe Hubert: Conceptualization, Project administration, Funding acquisition

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2021.114206.

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IV.5 Supplementary material for article II.2.1 – Supercritical fluid chromatography–mass spectrometry in routine anti-doping analyses: Estimation of retention time variability under reproducible conditions.

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Table S2

Mixture	Compound name	Concentration	Ionization mode
1	Ethisterone	10 ng/mL	ESI positive
	Letrozole	1 µg/mL	
	Niketamide metabolite	100 ng/mL	
	Betamethasone	1 µg/mL	
	Niketamide	10 ng/mL	
	Eplenerone	100 ng/mL	
	Furfenorex	10 ng/mL	
	Octopamine	1 µg/mL	
	Atenolol	10 ng/mL	
2	Methylphenidate metabolite	1 µg/mL	ESI positive
	Isometheptene	1 µg/mL	
	Etilefrine	100 ng/mL	
	Bromantane metabolite	100 ng/mL	
	JWH-250 metabolite 2	10 ng/mL	
	Fentanyl	10 ng/mL	
	Morphine	1 µg/mL	
	Clomiphene	1 µg/mL	
	Prednisolone	1 µg/mL	
	Clomiphene metabolite	1 µg/mL	
	Oxandrolone	100 ng/mL	
3	Oxilofrine	1 µg/mL	ESI positive
	JWH-250 metabolite 1	100 ng/mL	
	Amphetamine	100 ng/mL	
	Norfentanyl	10 ng/mL	
	Cocaine	10 ng/mL	
	Dexamethasone	1 µg/mL	
	Prednisone	1 µg/mL	
	Fluoxymesterone	100 ng/mL	

4	Amiloride	10 ng/mL	ESI positive
	Stanozolol metabolite	100 ng/mL	
	Metamphetamine	10 ng/mL	
	Benzoylecgonine	100 ng/mL	
	Propanolol	10 ng/mL	
	Tamoxifene	10 ng/mL	
	Fenfluramine	100 ng/mL	
	Fenbutrazate	10 ng/mL	
	Gestrinone	100 ng/mL	
5	Formoterol	100 ng/mL	ESI positive
	Methylecgonine	10 ng/mL	
	Terbutaline	10 ng/mL	
	Salbutamol	10 ng/mL	
	Benzylpiperazine	10 ng/mL	
	Methylphenidate	10 ng/mL	
	Timolol	10 ng/mL	
	Stanozolol	10 ng/mL	
	Trimetazidine	10 ng/mL	
6	Chlortalidone	1 µg/mL	ESI negative
	Bendroflumethiazide	1 µg/mL	
	Hydroclorothiazide	1 µg/mL	
	Etacrynic acid	1 µg/mL	
	Furosemide	1 µg/mL	
	Probenecide	1 µg/mL	

Table S3

Mix	Compound name	Precursor – Product (m/z)	CV (V)	CE (eV)
1	Ethisterone	337 – 190	44	18
	Letrozole	313.2 – 97	18	22
	Niketamide metabolite	151 – 80	40	32
	Betamethasone	393.1 - 373.1	18	20
	Niketamide	179.1 – 108.1	44	6
	Eplenerone	415.2 – 163.1	34	18
	Furfenorex	230 – 81	40	18
	Octopamine	136 – 91.1	40	16
	Atenolol	267.2 – 145.1	48	16
2	Methylphenidate metabolite	220.1 – 84.1	20	18
	Isometheptene	142.1 - 69.1	34	14
	Etilefrine	182.1 - 164.1	30	12
	Bromantane metabolite	322.1 - 91.1	16	32
	JWH-250 metabolite 2	352.3 - 121.1	50	22
	Fentanyl	337.2 - 188.1	38	20
	Morphine	286.1 - 152.9	66	38
	Clomiphene	406.2 - 71.9	25	25
	Prednisolone	361.2 - 325.1	6	8
	Clomiphene metabolite	418.2 - 72.1	25	25
	Oxandrolone	307.2 - 271.2	24	12
3	Oxilofrine	182.1 - 105.1	20	20
	JWH-250 metabolite 1	366.2 - 121.1	50	20
	Amphetamine	136.1-119.0 /91.0	25	8 ;14
	Norfentanyl	233.1 - 84.1	30	18
	Cocaine	304.1 - 182.1	44	18
	Dexamethasone	393.2 - 355.2	12	10
	Prednisone	359.1 - 313.1	30	12
	Fluoxymesterone	337.2 - 281.3	42	20

4	Amiloride	230 - 171	10	18
	Stanozolol metabolite	345.2 - 97	22	38
	Metamphetamine	150.1 - 91.1	18	16
	Benzoylecgonine	290.2 - 105.1	10	30
	Propanolol	260.1 - 116.1	24	20
	Tamoxifene	372.2 - 72.1	25	25
	Fenfluramine	232.1 - 159.1	38	22
	Fenbutrazate	368.2 - 191.1	20	22
	Gestrinone	309.1 - 241.1	42	24
5	Formoterol	345.1 - 149.1	10	18
	Methylecgonine	200.1 - 182.1	10	16
	Terbutaline	226.1 - 152.1	10	16
	Salbutamol	240.2 - 148.1	12	16
	Benzylpiperazine	177.1 - 91.1	10	18
	Methylphenidate	234.1 - 84	10	18
	Timolol	317.1 - 74.1	12	20
	Stanozolol	329.2 - 81.1	26	40
	Trimetazidine	267.1 - 181.1	18	10
6	Chlortalidone	337 - 190	12	18
	Bendroflumethiazide	420.1 - 289	50	22
	Hydroclorothiazide	296 - 269	50	20
	Etacrynic acid	303 - 245	8	18
	Furosemide	329 - 285; 205	10	16;20
	Probenecid	284.1 - 140	25	24

IV.6 Supplementary material for article II.2.2 – Ultra-high performance supercritical fluid chromatography coupled to tandem mass spectrometry for antidoping analyses: Assessment of the inter-laboratory reproducibility with urine samples.

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Analytical Science Advances (2020), 1-8.

Table S1: Stock solutions of each doping agents at two different concentration levels.

Name	**Concentration – lower (ng.mL^{-1})**	**Concentration – higher (ng.mL^{-1})**
Amiloride	5	50
Amphetamine	50	500
Atenolol	5	50
Benzoylecgonine	50	500
Fenbrutazate	5	50
Fentanyl	5	50
Fluoxymesterone	50	500
Gestrinone	5	50
Hydrochlorothiazide	50	500
JWH 205 met 1	5	50
Niketamide	5	50
Niketamide met	5	50
Norfentanyl	5	50
Prednisone	5	50
Probenecide	50	500
Propanolol	5	50
Salbutamol	5	50
Salbutamol-d5 (ISTD)	*5*	*50*
Stanozolol	5	50
Tamoxifene	5	50
Terbutaline	5	50
Trimetazidine	5	50

Table S2: Dilution steps performed for each blind urine from original samples.

Sample	Dilution step
Blind urine 1	Dilution x100
Blind urine 2	Dilution x10
Blind urine 3	Dilution x100
Blind urine 4	Dilution x10
Blind urine 5	Dilution x10
Blind urine 6	Dilution x10
Blind urine 7	Dilution x10

Table S3: Systems set-up used in this study.

Chromatographic system	Tandem mass spectrometer
Waters Acquity UPC^2 (BSM+SM+BPR+8-positions CM+PDA)	Waters Xevo TQ-S
Waters Acquity UPC^2 (BSM+SM+BPR+2-positions CM+PDA)	Waters Xevo TQ-XS
Waters Acquity UPC^2 (BSM+SM+BPR+2-positions CM+PDA)	Waters Xevo microTQ-S
Waters Acquity UPC^2 (BSM+SM+BPR+2-positions CM+PDA)	Waters Xevo TQ-S

Table S4: MS/MS parameters for each doping agent.

Name	ESI mode	Precursor (m/z)	Product (m/z)	Cone voltage (V)	Collision energy (eV)
Amiloride	POS	230	171	10	18
Amphetamine	POS	136	91	25	14
Atenolol	POS	267	145	48	28
Benzoylecgonine	POS	290	105	10	30
Fenbrutazate	POS	368	191	20	22
Fentanyl	POS	337	188	38	20
Fluoxymesterone	POS	337	281	42	20
Gestrinone	POS	309	241	42	24
Hydrochlorothiazide	NEG	296	269	50	20
JWH 205 met 1	POS	366	121	50	20
Niketamide	POS	179	108	44	18
Niketamide met	POS	151	80	40	20
Norfentanyl	POS	233	84	30	18
Prednisone	POS	359	313	30	12
Probenecide	NEG	284	140	25	24
Propanolol	POS	260	116	24	20
Salbutamol	POS	240	148	24	16
Stanozolol	POS	329	81	52	40
Tamoxifene	POS	372	72	25	25
Terbutaline	POS	226	152	20	16
Trimetazidine	POS	267	181	36	10
Salbutamol-d5 (ISTD)	POS - NEG	243 – 241	151 – 149	12	16

Table S5: Average RSD (%) values for intra-injection variability recorded by each laboratory.

Name	Lab 1	Lab 2	Lab 3	Lab 4	Average all labs
Amiloride	0.03%	0.00%	0.09%	0.00%	0.03%
Amphetamine	0.00%	0.00%	0.00%	0.03%	0.01%
Atenolol	0.07%	0.03%	0.07%	0.02%	0.05%
Benzoylecgonine	0.04%	0.00%	0.00%	0.03%	0.02%
Fenbrutazate	0.00%	0.33%	0.41%	0.75%	0.37%
Fentanyl	0.00%	0.00%	0.16%	0.05%	0.05%
Fluoxymesterone	0.04%	0.04%	0.00%	0.07%	0.04%
Gestrinone	0.04%	0.00%	0.04%	0.06%	0.04%
Hydrochlorothiazide	0.02%	0.03%	0.00%	0.04%	0.02%
JWH 205 met 1	0.00%	0.04%	0.14%	0.04%	0.05%
Niketamide	0.39%	0.09%	0.33%	0.67%	0.37%
Niketamide met	0.00%	0.05%	0.00%	0.02%	0.02%
Norfentanyl	0.00%	0.08%	0.00%	0.00%	0.02%
Prednisone	0.00%	0.04%	0.08%	0.08%	0.05%
Probenecide	0.04%	0.07%	0.07%	0.05%	0.05%
Propanolol	0.00%	0.00%	0.12%	0.03%	0.04%
Salbutamol	0.00%	0.03%	0.00%	0.02%	0.01%
Stanozolol	0.04%	0.12%	0.08%	0.00%	0.06%
Tamoxifene	0.00%	0.00%	0.00%	0.00%	0.00%
Terbutaline	0.00%	0.03%	0.00%	0.00%	0.01%
Trimetazidine	0.00%	0.00%	0.12%	0.00%	0.03%
Salbutamol-d5 (ISTD)	*0.00%*	*0.03%*	*0.03%*	*0.00%*	*0.02%*

IV.7 Supplementary material for article II.3.1 – Investigating the use of unconventional temperatures in supercritical fluid chromatography.

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Analytica Chimica Acta, 1134 (2020), 84-95.

1. Sample preparation procedures

Samples used for the theoretical aspects (butylparaben; uracil; maleic acid and indoxyl sulphate) were prepared according to the following procedure: an initial dilution to obtain stock solution at a concentration of 1 $mg.mL^{-1}$ was performed with either pure water or pure ACN. A second and final dilution, to a concentration of 200 $\mu g.mL^{-1}$, was made with pure ACN for all analytes.

Compounds used in the column stability study were first solubilized in either water (i.e. uracil, caffeine and adenosine) or acetonitrile for butylparaben, to prepare stock solutions at 1 $mg.mL^{-1}$. A mixture of these four analytes was then prepared, followed by a dilution to a final concentration of 200 $\mu g.mL^{-1}$ with pure acetonitrile as an aprotic injection solvent.

Compounds used in the second part of this study were dissolved, at a concentration of 1 $mg.mL^{-1}$, in either pure MeOH (i.e. dexamethasone, betamethasone, 2**β**-/19-/6**α**-/15**β**-/11**β**-/2**α**-/16**α**-hydroxytestosterone) or pure EtOH (i.e. E/Z-endoxifen). The three following mixtures were then prepared using pure ACN as sample diluent: dexamethasone and betamethasone, both at 50 $\mu g.mL^{-1}$; 2**β**-/19-/6**α**-/15**β**-/11**β**-/2**α**-/16**α**-hydroxytestosterone, at a concentration of 5 $\mu g.mL^{-1}$; 2**β**-hydroxytestosterone and 2**α**-hydroxytestosterone, both at 5 $\mu g.mL^{-1}$. E/Z-endoxifen was also diluted with pure ACN to obtain a sample concentration of 10 $\mu g.mL^{-1}$.

Paroxetine hydrochloride anhydrous and its pharmacopeia impurities A, C and H were each dissolved in water at a concentration of 1 $mg.mL^{-1}$. A mixture was then prepared with all four components to have paroxetine hydrochloride anhydrous at a concentration of 50 $\mu g.mL^{-1}$, while its impurities were at a concentration of 10 $\mu g.mL^{-1}$(dilution performed with pure ACN), necessary to obtain a S/N ratio of approximately 10 when performing the SFC analysis at a column temperature of 40°C.

2. Chromatographic conditions

In sections 3.1 to 3.3, isocratic conditions have been applied to all analyses. The cosolvent used was a mixture of 95/5 MeOH/water *v/*v for butylparaben and uracil, while for maleic acid and indoxyl sulphate an addition of 10 mM of ammonium formate in the cosolvent was made.

In section 3.4, the following profile was employed: an initial isocratic hold, of 0.5 min was kept with a mobile phase ratio of 98/2 CO_2/cosolvent *v/v*, with the cosolvent being a mixture of 95/5 MeOH/water *v/v* + 10 mM of ammonium formate. Then, a gradient was applied reaching up to 40% of co-solvent in 4 min, followed by a second isocratic step at 60:40 CO_2/co-solvent *v/v* for 0.5 min. The initial conditions were then re-established for a total run time of 5 min. UV detection was performed at 254 nm. Flow-rate was set at 2.0 mL.min^{-1}.

In section 3.5, various conditions were used. For the analysis of dexamethasone/betamethasone and for the mixture of the 7 hydroxytestosterone samples, the same gradient profile was used at 80°C and 40°C, namely 2 to 40% of co-solvent in 4 min, followed by an isocratic hold at 60/40 CO_2/B *v/v* for 0.5 min, then return to initial conditions for a total run time of 5.5 min. At 5°C and -5°C, a slightly different gradient was used: 2 to 35% of cosolvent in 3.5 min, followed by an isocratic hold at 65/35 CO_2/B *v/v* for 0.5 min, then return to initial conditions for a total run time of 5.5 min. Flow-rate was set at 1.2 mL.min^{-1}. Single ion recording (SIR) MS detection was performed at 393 *m/z* for dexamethasone/betamethasone and 305 *m/z* for the hydroxytestosterone samples mixture. The co-solvent (mobile phase B) was a mixture of 95/5 MeOH/H2O *v/v* + 10 mM of ammonium formate.

The conditions used for the separation of the 2**β**-/2**α**-hydroxytestosterone mixture were as follow: at all tested temperatures (i.e. 40°C, 5°C and -5°C), a gradient mode was used. An initial ramp from 45 to 75% of co-solvent was reached in 6 min, with a subsequent isocratic step at 75% for 0.5 min. The initial conditions were reached afterwards for a total run time of 9 min. Flow-rate was set at 0.5 mL.min^{-1}. Single ion recording (SIR) MS detection was performed at 305 *m/z*. For (E/Z)-endoxifen, the following conditions were used: at all tested temperatures

(i.e. 80°C, 40°C, 5°C and -5°C), a gradient mode was used. An initial ramp from 25 to 65% of organic modifier was reached in 5.5 min, with a subsequent isocratic step at 65% for 0.5 min. The initial conditions were reached afterwards for a total run time of 9 min. Flow-rate was set at 0.7 $mL.min^{-1}$. Single ion recording (SIR) detection was performed at 374 *m/z*. The co-solvent was a mixture of 95/5 MeOH/H2O *v/v* + 10 mM of ammonium formate.

The analysis of paroxetine and its main impurities (paragraph 3.6) was performed using the following conditions: the same gradient profile was used at 80 and 40°C: 2 to 40% of co-solvent in 4 min, followed by a isocratic hold at 60/40 CO_2/B *v/v* for 0.5 min, then return to initial conditions for a total run time of 5.5 min. At 5°C and -5°C, a slightly different gradient was used: 2 to 35% of cosolvent in 3.5 min, followed by an isocratic hold at 65/35 CO_2/B *v/v* for 0.5 min, then return to initial conditions for a total run time of 5.5 min. Flow-rate was set at 1.2 $mL.min^{-1}$. UV detection was performed at 214 nm. The cosolvent was 95/5 MeOH/H2O *v/v* + 0.2% of ammonia.

3. Extra-column system dispersion

3.1 Determination of system's extra-column volume

The residence time of test solute (elution time of butylparaben) was measured at various flow rates (in absence of column). The system volume was determined from the relationship between residence time and reciprocal flow rate. The system's extra-column volume was determined as Vec = 56 and 72 µL with the SFC and external oven, respectively.

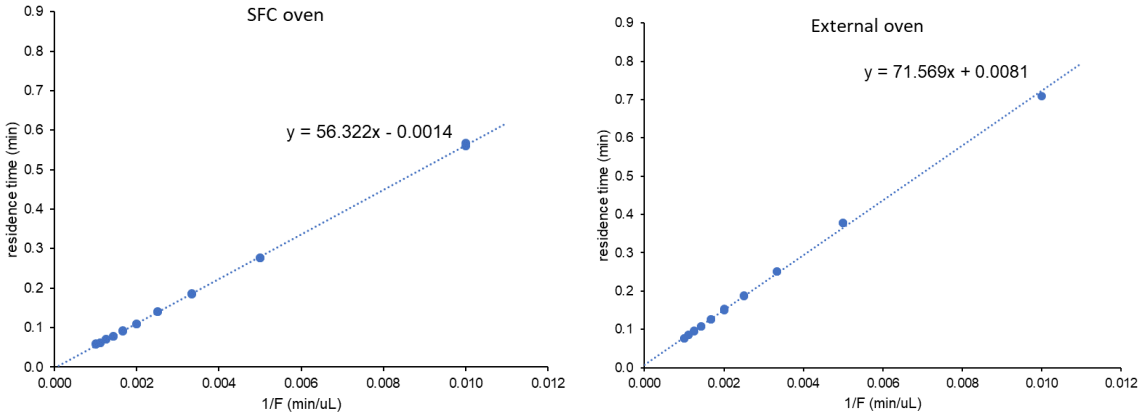


Figure S1: Determination of extra-column system volume. The slope of the fitted curves corresponds to the system volume (V_{ec} = 56 µL for the system equipped with the SFC oven, while V_{ec} = 72 µL for the system equipped with the external oven.)

3.2 Determination of extra-column band dispersion

The spatial peak variance can be determined from the peak width measured at half height ($w_{1/2}$):

$$\sigma_{ec}^2 = \mathrm{F}^2 \frac{w_{1/2\_ec}^2}{5.545}$$

where *F* is the flow rate and *ec* in subscript corresponds to extra-column (measured in the absence of the column). Then plate height values can be corrected for the contributions of the extra-column volume using the following equation:

$$H = \mathrm{L} \frac{w_{1/2}^2 - w_{1/2\_ec}^2}{5.545(t_r - t_e)^2}$$

where $w_{1/2}$ is the peak width measured with column, and t_r and t_e are the retention and elution times (at peak apex) of the test compounds obtained with and in absence of the column (respectively).

On the other hand, peak variance can also be determined from the central moments (μ) of a peak:

$$\mu_1 = \frac{\int Ce\,(t) t\; dt}{\int Ce\,(t)\; dt}$$

$$\mu_2 = \frac{\int Ce\,(t)(t-\mu_1)^2\; dt}{\int Ce\,(t)\; dt}$$

where Ce(*t*) is the concentration of the sample compound in the mobile phase leaving from the column as a function of time (*t*). The values of μ_1 and μ_2 are calculated by integrating the elution peak profile. The first moment gives the elution time while μ_2 is equal to the variance of the peak.

In order to take into consideration the extra-column system dispersion, measurements were performed by replacing the column with a zero dead volume (ZDV) union connector and injecting butylparaben as model analyte to estimate extra-column band broadening (variance). Injections were performed under the same conditions as those made with columns, at all the flow-rates, temperatures and co-solvent proportions previously tested. Plate heights were then corrected for extra-column peak dispersion. The extra-column volumes of the two system configurations (original or external oven) were measured under LC conditions, using 100% MeOH as mobile phase, to avoid problems related to the mobile phase compression. Extra-column dispersion strongly depended on the conditions. Spatial variance between 20 and 170 µL2 was observed. The moment method resulted in significantly higher variance values than the half-height method. Probably, the latter one underestimates the true variance (since peaks were not perfectly symmetrical). Thus, the values obtained with the moment method were considered.

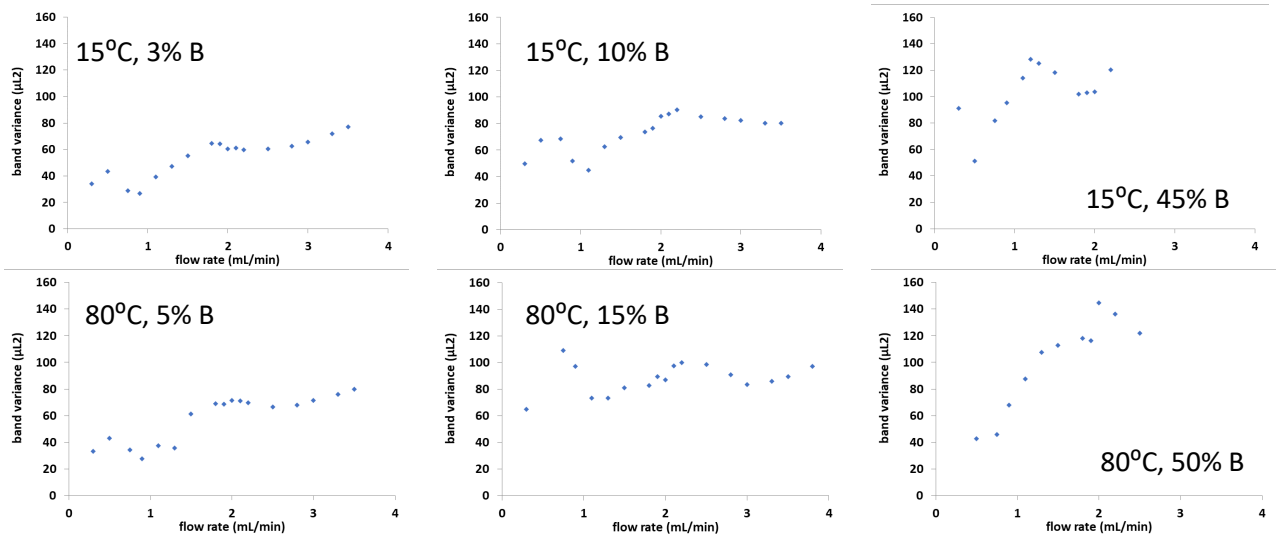


Figure S2: Plots of spatial band variance *vs* flow rate. Representative examples (15 and 80ºC, and various mobile phase composition) on the determination of extra-column band broadening. Test solute: butylparaben. Extra-column band variance was determined in the same way for all conditions set in the study.

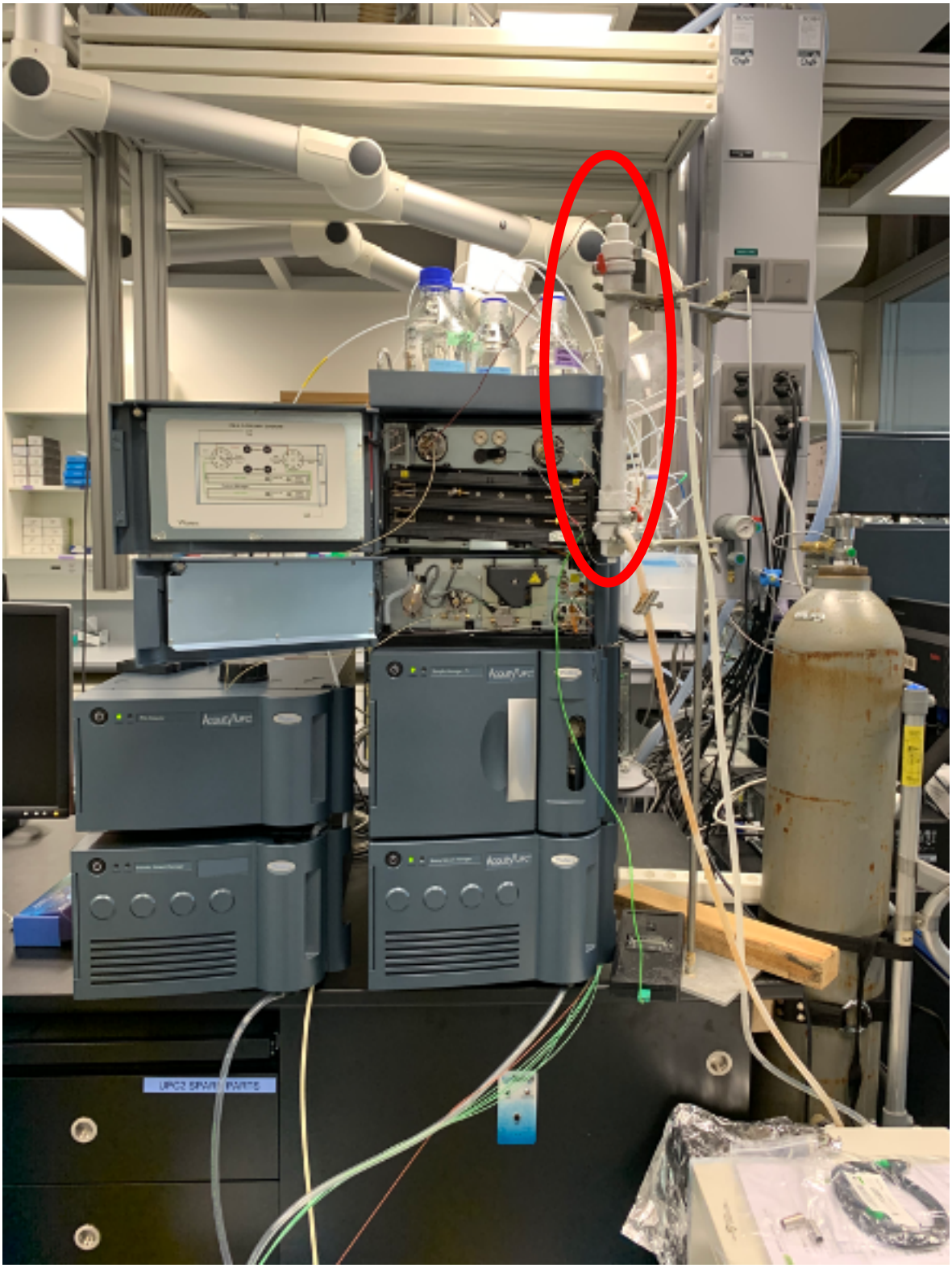


Figure S3: Experimental setup of the UHPSFC chromatographic system with the external oven to reach sub-zero temperature environment. The column was fitted inside the cylindric container (highlighted with red circle).

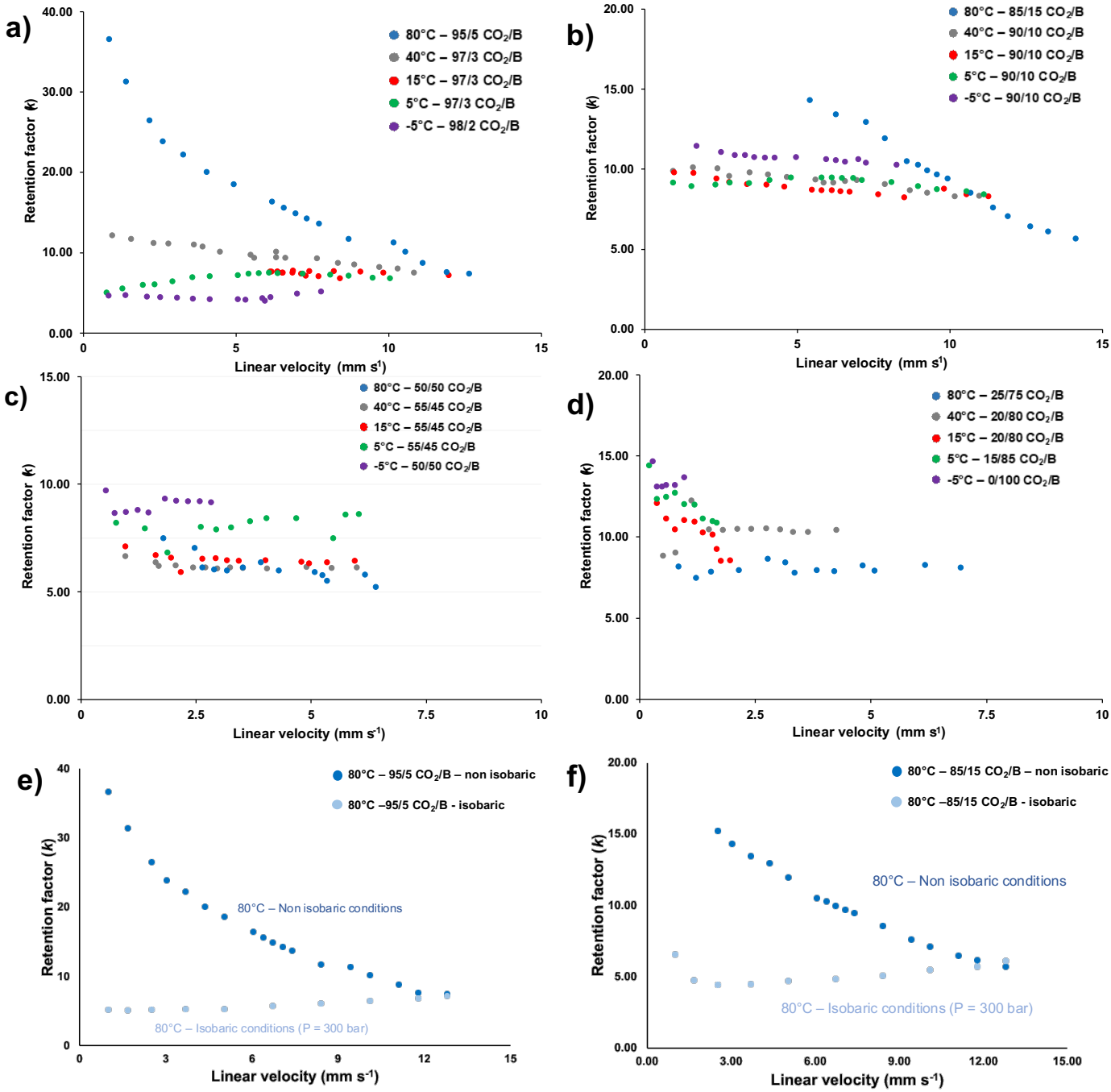


Figure S4: *k vs u* plots obtained for a) butylparaben, b) uracil, c) maleic acid and d) indoxyl sulphate at different temperatures and cosolvent percentages. Figures S4e and S4f represent the *k vs u* plot relative, respectively, to butylparaben and uracil at 80°C in isobaric and non-isobaric conditions.

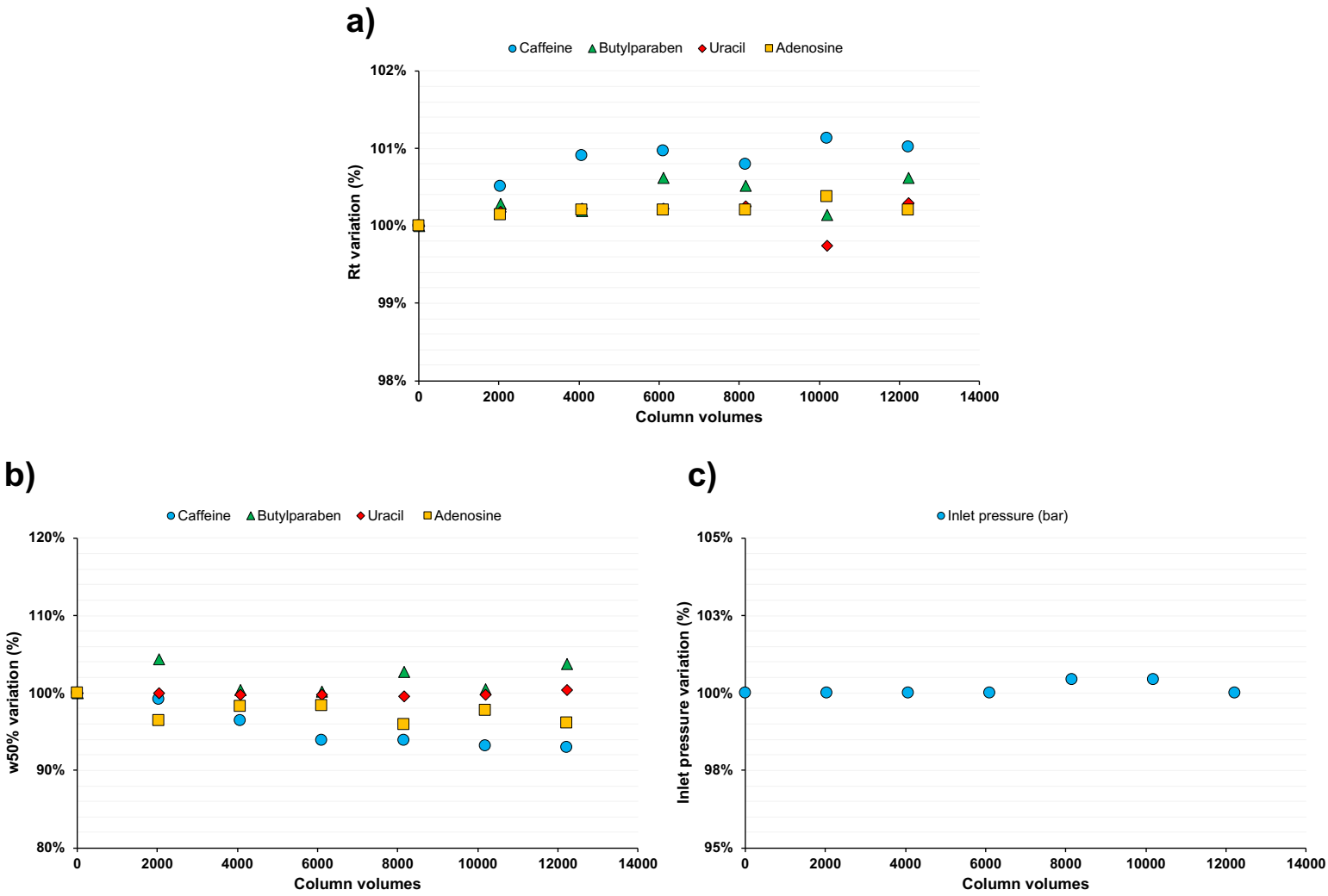


Figure S5: a) Variation (%) of the retention times for the four analytes, recorded at different mobile phase volumes flushed through the column. b) Variation (%) of the peak width, measured at half-height, for the four analytes at increasing column volumes. c) Variation (%) of the inlet column pressure recorded at increasing column volumes.

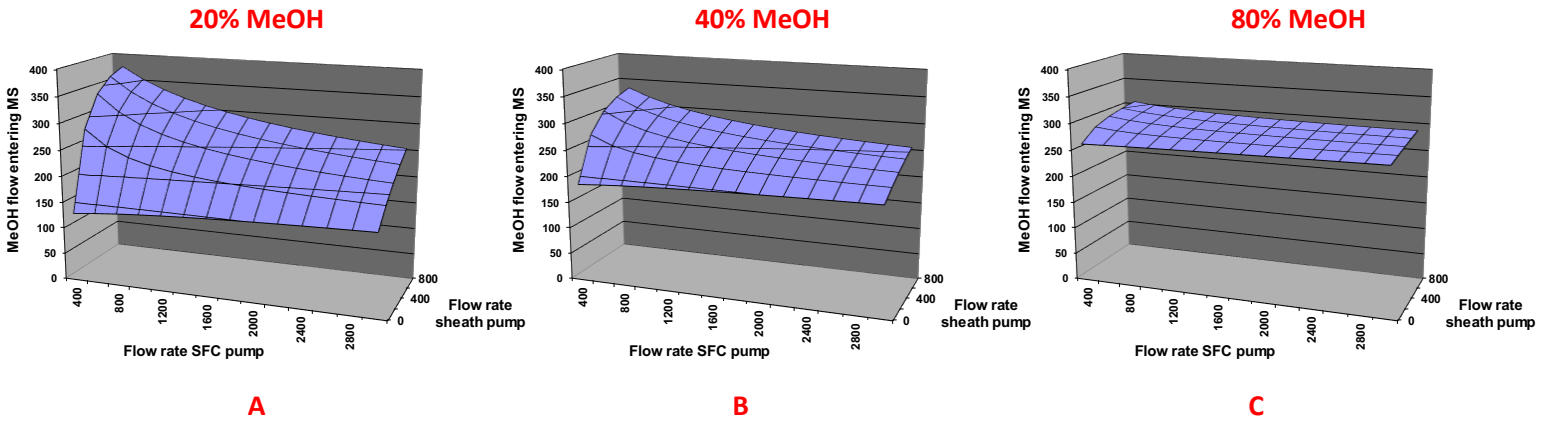
IV.8 Supplementary material for article II.4.1 – Applicability of supercritical fluid chromatography-mass spectrometry to metabolomics I – Optimization of separation conditions for the simultaneous analysis of hydrophilic and lipophilic substances.

Authors: Vincent Desfontaine[a], Gioacchino Luca Losacco[a], Yoric Gagnebin[a], Juilian Pezzatti[a], William P. Farrell[b], Victor Gonzalez-Ruiz[a], Serge Rudaz[a], Jean-Luc Veuthey[a], Davy Guillarme[a]

(a) School of Pharmaceutical Sciences, University of Geneva, CMU – Rue Michel-Servet 1, Geneva 4 1211, Switzerland.

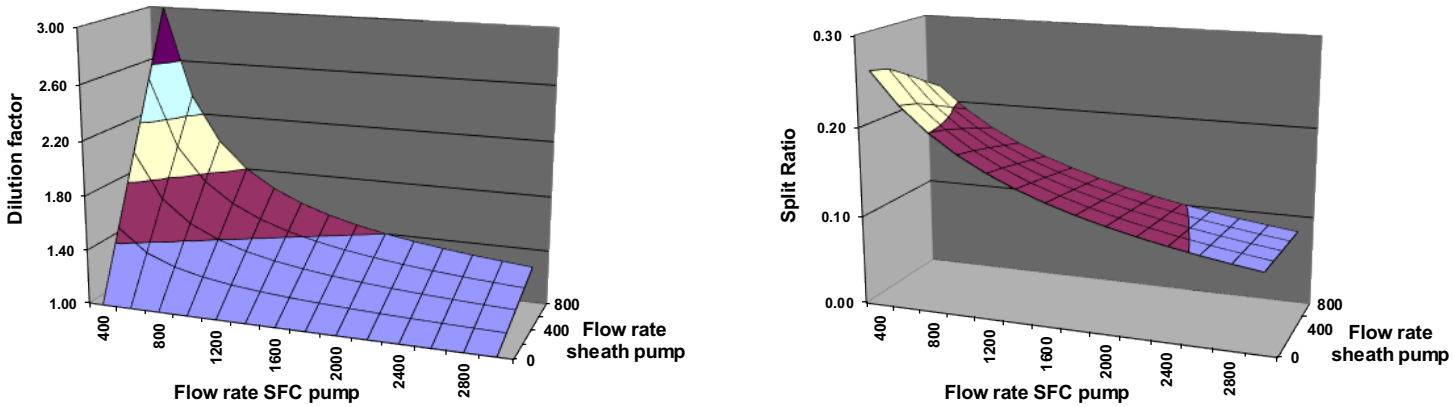
(b) Pfizer, Inc., Worldwide Medicinal Chemistry, La Jolla Laboratories, 10770 Science Center Drive, San Diego, CA 92121, USA.

Journal of Chromatography A, 1562 (2018), 96-107.



150 bar, 40°C, Waters Interface

Figure S1: Calculation of the amount of MeOH entering into the MS as a function of the SFC pump and the make-up pump flow rates, with the Waters *"pre-BPR splitter with sheath pump"* interface, using different mobile phase conditions: CO_2/MeOH 80:20 (A), CO_2/MeOH 60:40 (B) and CO_2/MeOH 20:80 (C). Mobile phase temperature of 40°C and backpressure of 150 bar



150 bar, 40°C, 80% MeOH
Waters Interface

.Figure S2: Modelization of the mobile phase dilution factor caused by the make-up flow for the Waters *"pre-BPR splitter with sheath pump"* interface as a function of SFC mobile phase flow rate (x-axis) and make-up pump flow rate (y-axis), for a mobile phase consisting of CO_2/MeOH (20:80). Mobile phase temperature of 40°C and backpressure of 150 bar.

IV.9 Supplementary material for article II.4.2 – Applicability of supercritical fluid chromatography-mass spectrometry to metabolomics II – Assessment of a comprehensive library of metabolites and evaluation of biological matrices.

Authors: Gioacchino Luca Losacco[a], Omar Ismail[b], Juilian Pezzatti[a], Victor Gonzalez-Ruiz[a], Julien Boccard[a], Serge Rudaz[a], Jean-Luc Veuthey[a], Davy Guillarme[a]

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Table S1

Mixture - ESI mode	Name	ME (%) – URINE	ME (%) – PLASMA	RSD (%) – STDs	RSD (%) – URINE	RSD (%)- PLASMA
1 – NEG	Taurine	70%	80%	0.33%	0.25%	0.00%
	2-Methylglutaric acid	47%	136%	0.34%	0.28%	0.36%
	Raffinose	80%	123%	0.29%	0.23%	0.30%
	Allantoin	63%	100%	0.33%	0.15%	0.19%
	Tryptophan	78%	116%	0.44%	0.25%	0.45%
	Uracil	102%	101%	0.34%	0.47%	0.19%
	Phosphoserine					
	3-dehydroshikimic acid	70%	129%	0.45%	0.18%	0.34%
	Thymidine monophosphate	43%	88%	0.26%	0.24%	0.54%
	Desthiobiotin	78%	89%	0.30%	0.31%	0.17%
2 – NEG	Ribitol = Adonitol	62%	60%	0.19%	0.62%	0.33%
	Glutathione	121%	115%	0.25%	0.33%	0.26%
	Glutamic acid	138%	109%	0.31%	0.33%	0.50%
	Lactose	87%	104%	0.34%	0.41%	0.29%
	Maleic acid	51%	59%	0.34%	0.32%	0.26%
	Glutaric acid	56%	82%	0.48%	0.36%	0.45%
	Indoxyl sulphate	89%	118%	0.26%	0.34%	0.32%
	Folic acid	54%	36%	0.09%	0.28%	0.32%
	Biotin	63%	60%	0.27%	0.32%	0.39%
	Uric acid	56%	25%	0.24%	0.39%	0.15%
3 – NEG	trans-Cinnamate	105%	92%	0.36%	0.40%	0.54%
	Rosmarinic acid	124%	155%	0.28%	0.26%	0.44%
	Melatonine	94%	95%	0.25%	0.24%	0.13%
	Oleic acid	91%	97%	0.25%	0.25%	0.13%
	Bilirubin	75%	115%	0.37%	0.53%	0.59%
4 – POS	Citrulline	45%	87%	0.20%	0.45%	0.83%
	Leucine	15%	25%	0.31%	0.20%	0.62%
	Adenosine	69%	89%	0.40%	0.22%	0.35%
	Trigonelline	101%	125%	0.32%	0.46%	0.59%
	Betaine	92%	149%	0.33%	0.43%	0.53%
	Riboflavin	10%	45%	0.21%	0.47%	0.48%
	Phosphorylethanolamine	13%	183%	0.45%	0.30%	0.62%
	Spermidine	49%	99%	0.25%	0.35%	0.37%
	Xanthurenic acid	17%	75%	0.37%	0.48%	0.99%
5 – POS	Picolinic acid	104%	345%	0.36%	0.15%	0.76%
	Lysine	132%	179%	0.47%	0.21%	0.67%
	Histidinol	83%	102%	0.58%	0.31%	0.73%
	Acetylcholine	124%	159%	0.37%	0.33%	0.69%
	Adenosine triphosphate	106%	97%	0.39%	0.44%	0.57%
	Adenosine monophosphate	93%	189%	0.50%	0.31%	0.77%
	Kynurenine	35%	73%	0.39%	0.28%	0.76%
	Adenine	75%	115%	0.37%	0.08%	0.52%
	Epinephrine	55%	98%	0.39%	0.29%	0.56%
6 - POS	Caffeine	125%	153%	0.30%	0.28%	0.30%
	Sphingomyelin	5%	62%	0.32%	0.47%	0.57%
	Lauroylcarnitine	68%	173%	0.56%	0.45%	0.62%

	Nicotine	117%	115%	0.39%	0.30%	0.41%
	Sphinganine	21%	103%	0.23%	0.69%	0.34%
	Retinyl palmitate	47%	171%	0.52%	1.19%	1.02%

IV.10 Supplementary material for article II.5.1 – Expanding the range of sub/supercritical fluid chromatography: Advantageous use of methanesulfonic acid in water-rich modifiers for peptide analysis.

Authors: Gioacchino Luca Losacco[a,b], Jimmy Oliviera DaSilva[c], Jinchu Liu[c], Erik L. Regalado[c], Jean-Luc Veuthey[a,b], Davy Guillarme[a,b]

(a) School of Pharmaceutical Sciences, University of Geneva, CMU – Rue Michel-Servet 1, Geneva 4 1211, Switzerland.

(b) Institute of Pharmaceutical Sciences of Western Switzerland, University of Geneva, CMU – Rue Michel-Servet 1, Geneva 4 1211, Switzerland.

(c) Analytical Research and Development, MRL, Merck & Co, Inc., 126 E. Lincoln Avenue, Rahway, NJ 07065, USA.

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1. Materials and methods

1.1 Sample preparation and stress procedure

At University of Geneva, the synthetic peptides 1N, 2N, 1B, 2B, 1A, 2A, 6mer, 9mer, 12mer, 15mer, 18mer and 21mer have all been prepared according to the following procedure: stock solutions for each peptide were made at 2 $mg.mL^{-1}$ using a mixture of ACN/H_2O 50:50 *v/v*. From the stock solutions, dilutions to 500 $\mu g.mL^{-1}$ in ACN/H_2O 50:50 *v/v* for each peptide were performed for samples subsequently tested in either UHPSFC-UV or UHPLC-UV conditions.

Liraglutide, leuprorelin, glucagon and eptifibatide samples were all solubilized to create stock solutions in pure water at a concentration of 2 $mg.mL^{-1}$. Linaclotide was initially solubilized in water at a concentration of 2 $mg.mL^{-1}$, followed by agitation at 300 rpm for 30 min using a ThermoMixer equipped with a 24x0.5 mL ThermoBlock from Eppendorf (Vaudaux-Eppendorf AG, Basel, Switzerland) to ensure a full solubilization of the peptide. Cyclosporin A was diluted at a concentration of 2 $mg.mL^{-1}$ in pure ACN.

Using stock solutions for each commercially available peptide, control samples have been prepared by dilution to a final concentration of 300 $ug.mL^{-1}$ in ACN/H_2O 50:50 *v/v*, following by an agitation at 300 rpm for one hour. Different stressing procedures have been employed for all commercial peptides. Exposure to basic conditions was performed by diluting the stock solutions with a mixture of ACN/0.1M NaOH aqueous solution 50:50 *v/v* at a final concentration of 300 $ug.mL^{-1}$, followed by agitation at 300 rpm for one hour. Stressing procedure in acidic conditions was performed by diluting the stock solutions with a mixture of ACN/0.1M HCl aqueous solution 50:50 *v/v* at a final concentration of 300 $ug.mL^{-1}$, followed by agitation at 300 rpm for one hour. Oxidative stress was performed by diluting the stock solutions with a mixture of ACN/30% hydrogen peroxide (H_2O_2) solution 50:50 *v/v* at a final concentration of 300 $ug.mL^{-1}$, followed by agitation at 300 rpm for one hour.

The crude mixture of the proprietary cyclic peptide (Merck & Co., Inc., Kenilworth, NJ, USA) was dissolved in 100% of methanol at a concentration of 1 $mg.mL^{-1}$.

1.2 Chromatographic instrumentation, data treatment and MS conditions

All UHPSFC-UV-MS analyses at the University of Geneva have been performed on a Waters Acquity UPC^2 system (Waters, Milford, MA, USA) equipped with a Binary Solvent Manager delivery pump, a Sample Manager autosampler which included a 10 µL loop for partial loop injection, a column oven with active preheater, a PDA detector with an 8.4 µL flow-cell and a two-step (active and passive) backpressure regulator (BPR). Such configuration was utilized for UHPSFC-UV analyses. A slightly different configuration was used, consisting in the removal of the PDA detector when the UHPSFC instrument was coupled to the MS detector to limit band broadening due to extra-column volume. The Waters Acquity UPC^2 system was hyphenated to a Waters TQD Triple Quadrupole mass spectrometer, fitted with a Z-spray ESI source, via a "*pre-BPR splitter with make-up pump"* SFC-MS interface provided by Waters. Make-up solvent was delivered by a Waters Acquity Isocratic Solvent Manager (ISM) module, at a flow-rate of 0.1 mL.min^{-1}. A mixture of MeOH/H_2O 95:5 *v/v* was chosen as the make-up solvent. The autosampler temperature was fixed at 8°C. Column temperature was set at 55°C. Injection volume was fixed at 1.0 µL. Mixtures of ACN/H_2O 75:25 *v/v* and 50:50 were used, respectively, as weak and strong needle washes. For UV detection, chromatograms were obtained at 214 nm using the "absorbance-compensated" mode, with the compensation range fixed between 350 – 450 nm. Empower v3.0 (Waters, Milford, MA, USA) was used to control the UHPSFC-UV system, while MassLynx v4.1 (Waters, Milford, MA, USA) was employed to handle the UHPSFC-MS instrument.

UHPLC-UV analyses have been performed on a Waters Acquity UPLC H-class system (Waters, Milford, MA, USA), equipped with a Quaternary Solvent Manager delivery pump, a Sample Manager autosampler, a column oven with active preheater and a PDA detector with a 500 nL flow-cell. A second UHPLC instrument, consisting in a Waters Acquity UPLC I-Class system equipped with a Binary Solvent Manager, an autosampler and a thermostatic column compartment, was hyphenated to the same Waters TQD Triple Quadrupole mass spectrometer as previously described to perform UHPLC-MS analyses. The autosampler

temperature was fixed at 8°C. Column temperature was set at 55°C. Injection volume was set at 0.5 µL. Mixtures of ACN/H_2O 5:95 *v/v* and 50:50 were used, respectively, as weak and strong needle washes. For UV detection, chromatograms were obtained at 214 nm. Empower v3.0 (Waters, Milford, MA, USA) was used to control the UHPLC-UV system, while MassLynx v4.1 (Waters, Milford, MA, USA) was employed to handle the UHPLC-MS instrument. Data treatment was performed via Microsoft Excel 2019. For both UHPSFC-MS and UHPLC-MS analyses, the same ionization conditions were used, consisting in a capillary voltage of +1.0 kV, cone voltage of 30 V, source temperature at 150°C and desolvation temperature at 500°C, desolvation and cone gas flows were set at 900 L/h and 50L/h, respectively. Nitrogen (N_2) was used as both desolvation and cone gas. All MS analyses were performed in ESI positive mode in both UHPSFC and UHPLC conditions. With both chromatographic techniques, a MS full scan mode was utilized. Each sample was injected twice, performing firstly a MS full scan with a m/z range from 500 to 1500, then a second MS full scan analysis with a m/z range from 250 to 750. Scan time was fixed at 0.25 min in all conditions.

Chiral SFC screening and optimization experiments were carried out on Waters Acquity UPC^2 (Waters Corp., Milford, MA, USA) equipped with a Binary Solvent Manager delivery pump, a Sample Manager autosampler which included a 10 µL loop for partial loop injection, a column oven with active preheater, a PDA detector with an 8.4 µL flow-cell and a two-step (active and passive) backpressure regulator (BPR). The system was coupled to a single quadrupole MS detector (Water Acquity QDa) via the same SFC-MS interface as previously described. Make-up solvent was delivered by a Waters Acquity Isocratic Solvent Manager (ISM) module. MassLynx software v4.1 was used for system control.

The preparative SFC instrumentation used in these studies was a Berger Multigram 3 (MG3). The instrument consists of an automatic nozzle back pressure regulator (BPR), a CO2 pump, a modifier pump, a cavro injector with loop, a column oven, a Knauer UV detector, an open bed cabinet fraction collector and ProNToTM software which controls the instrument and data processing.

Figure S1: Comparison of 0.2% NH_4OH *vs* 0.1% TFA on the Torus 2-PIC for peptide 1B and 2A.

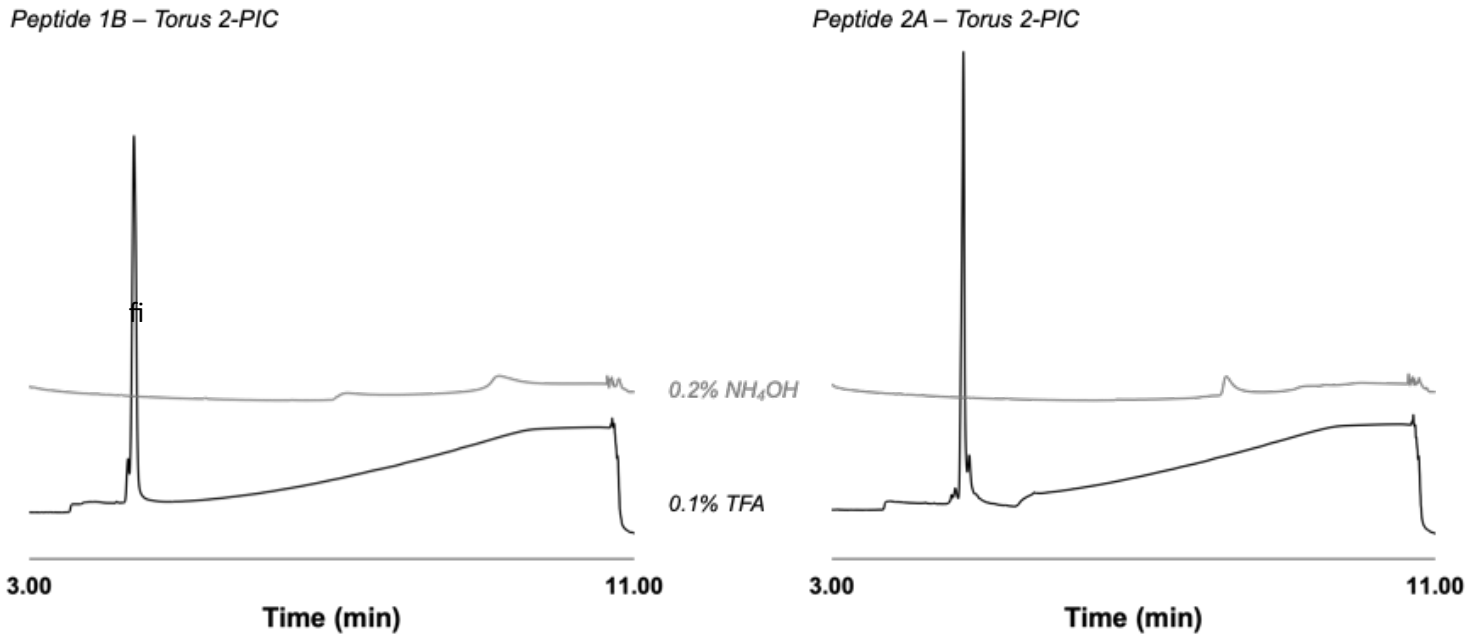

Figure S2: Comparison of 0.05% and 0.1% of MSA and TFA on the Torus 2-PIC for peptide 2N.

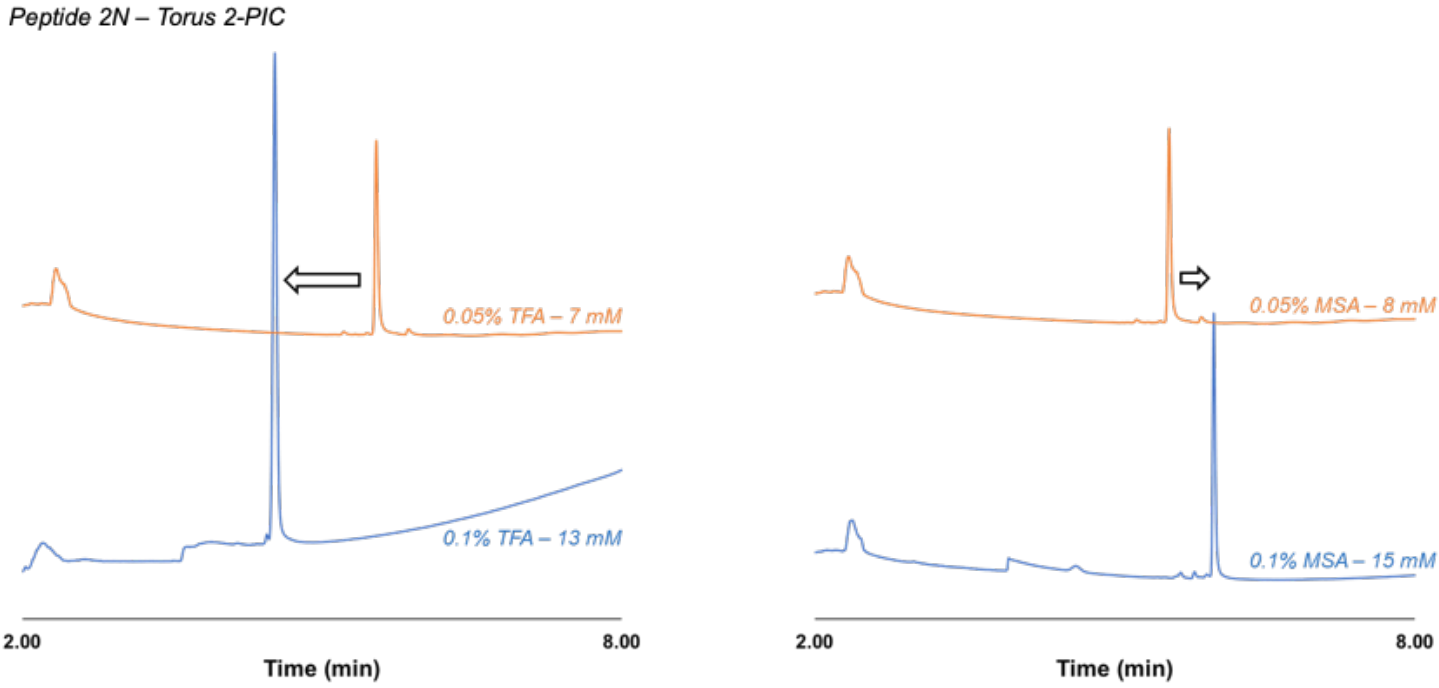

Figure S3: Chromatograms of commercial peptides under UHPSFC conditions.

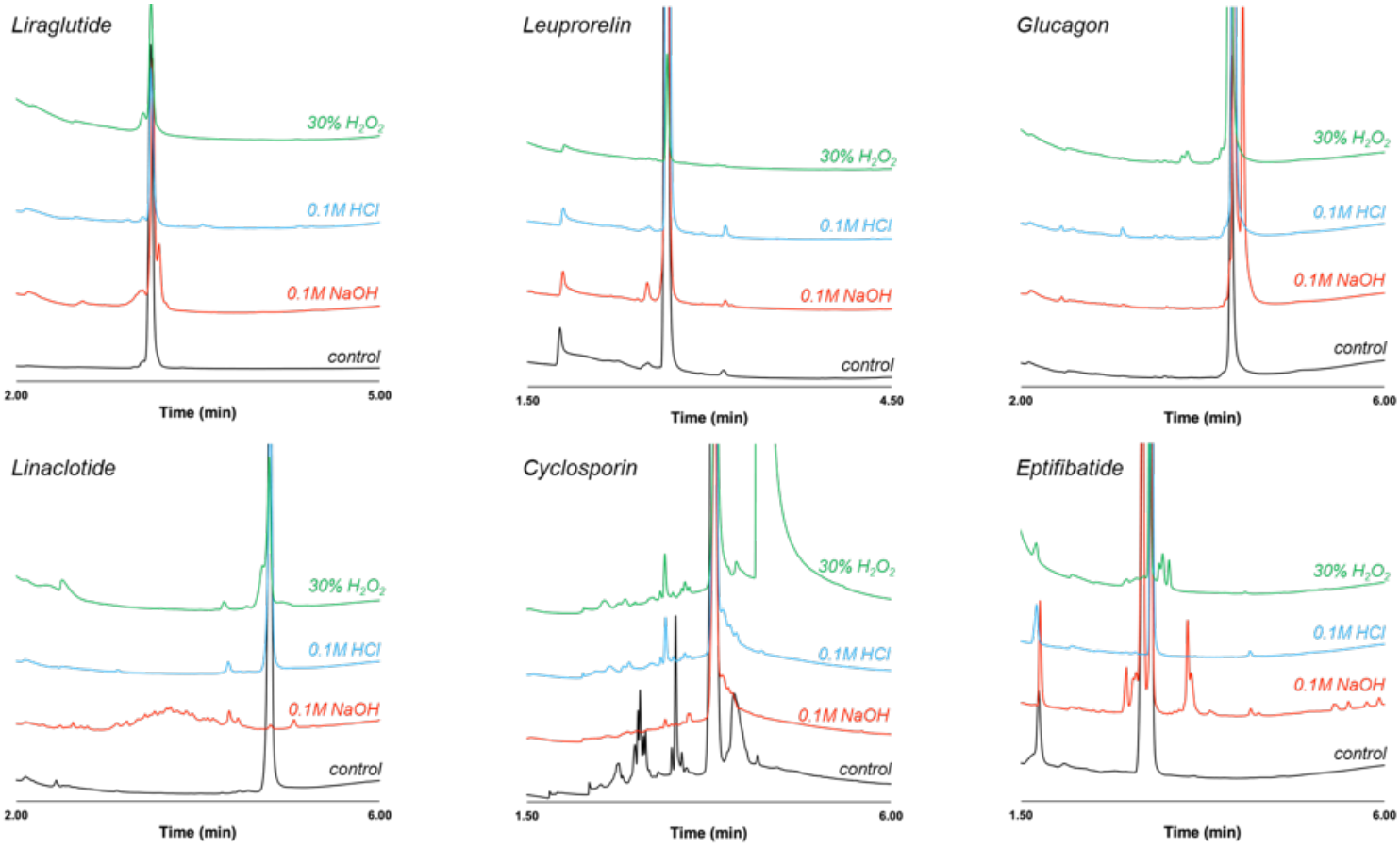

Figure S4: Chromatograms of commercial peptides under UHPLC conditions.

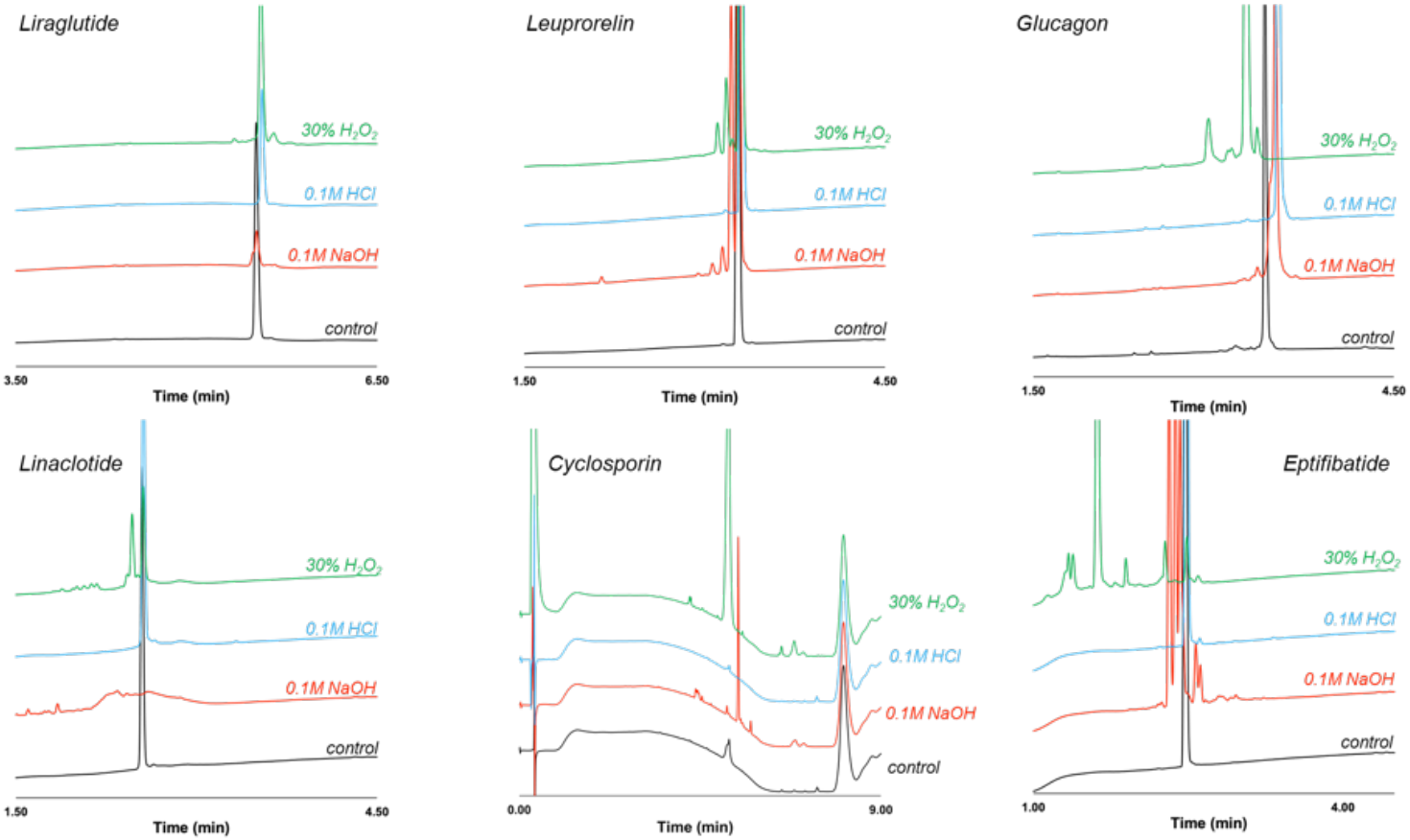

Figure S5: Chromatograms of eptifibatide and eptifibatide + impurities after stressing procedures in UHPSFC-UV-MS and UHPLC-UV-MS conditions.

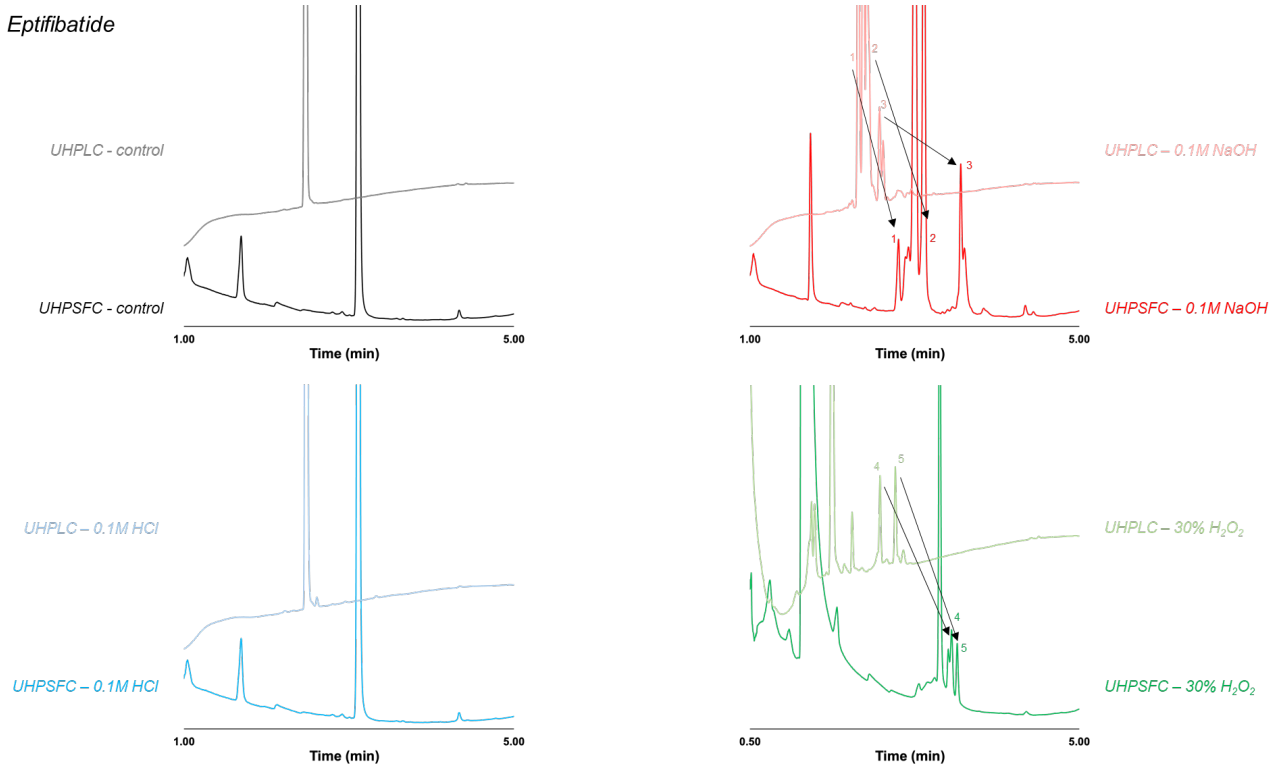

Table S1: Retention times of lysine, arginine, aspartic and glutamic acid with 0.05% and 0.1% of MSA and TFA.

Retention times				
	Lysine	Arginine	Glutamic acid	Aspartic acid
0.1% MSA	3.04	3.25	2.90	2.98
0.05% MSA	2.95	3.11	2.93	2.96
0.1% TFA	2.77	2.88	3.09	2.87
0.05% TFA	2.81	2.94	3.15	2.91