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 SFCHRMS 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 SFCHRMS 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 [...]

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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

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 (CO_2) 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 di-

lutions 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/H2O 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 $50\% \leq ME \geq 150\%$ 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 ($50\% \leq ME \geq 150\%$), ion suppression ($50\% \leq ME$) and ion enhance-
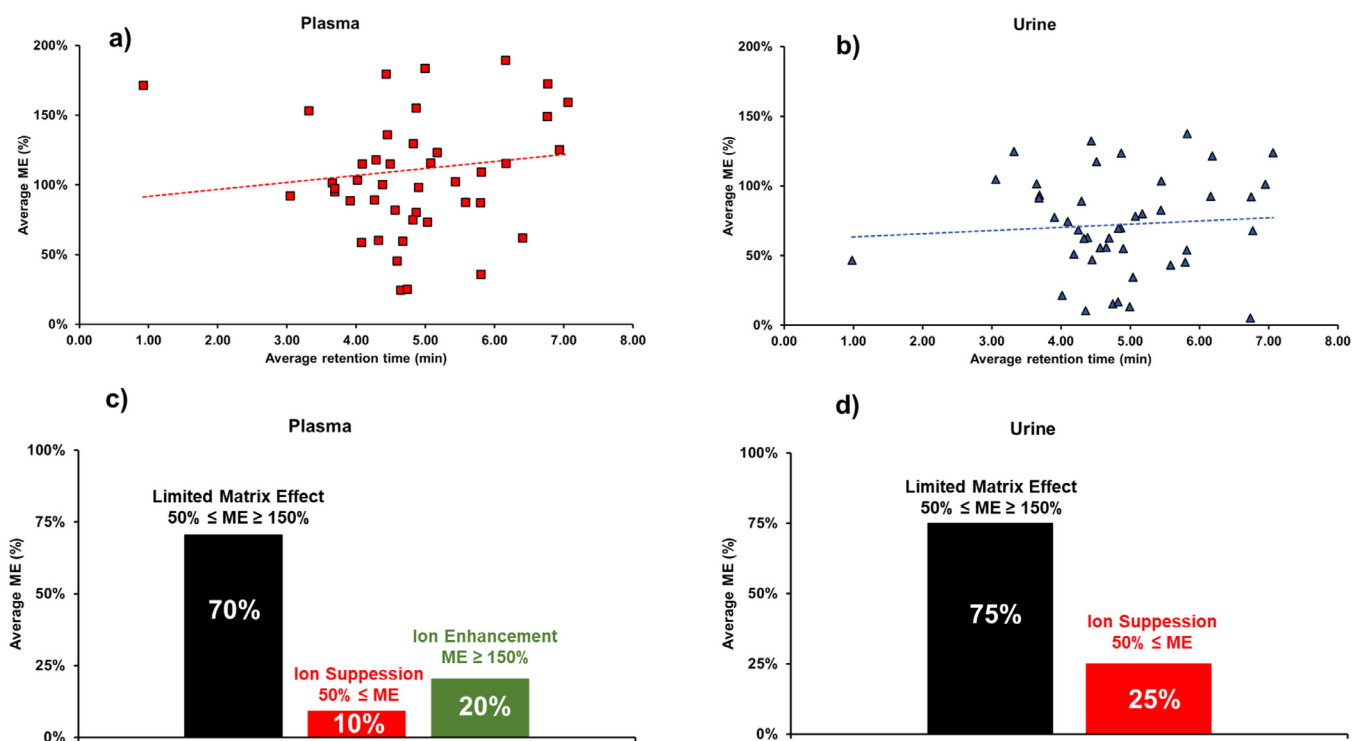

Fig. 1. (A) Scatter plot of the average matrix effect (%) as a function of the average retention time (min) for each metabolite in plasma. (B) Scatter plot of the average matrix effect (%) as a function of the average retention time (min) for each metabolite in urine. (C) Bar graph showing the distribution 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 ≥ 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 ≤ 50%) or limited matrix effect (50% ≤ ME ≥ 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
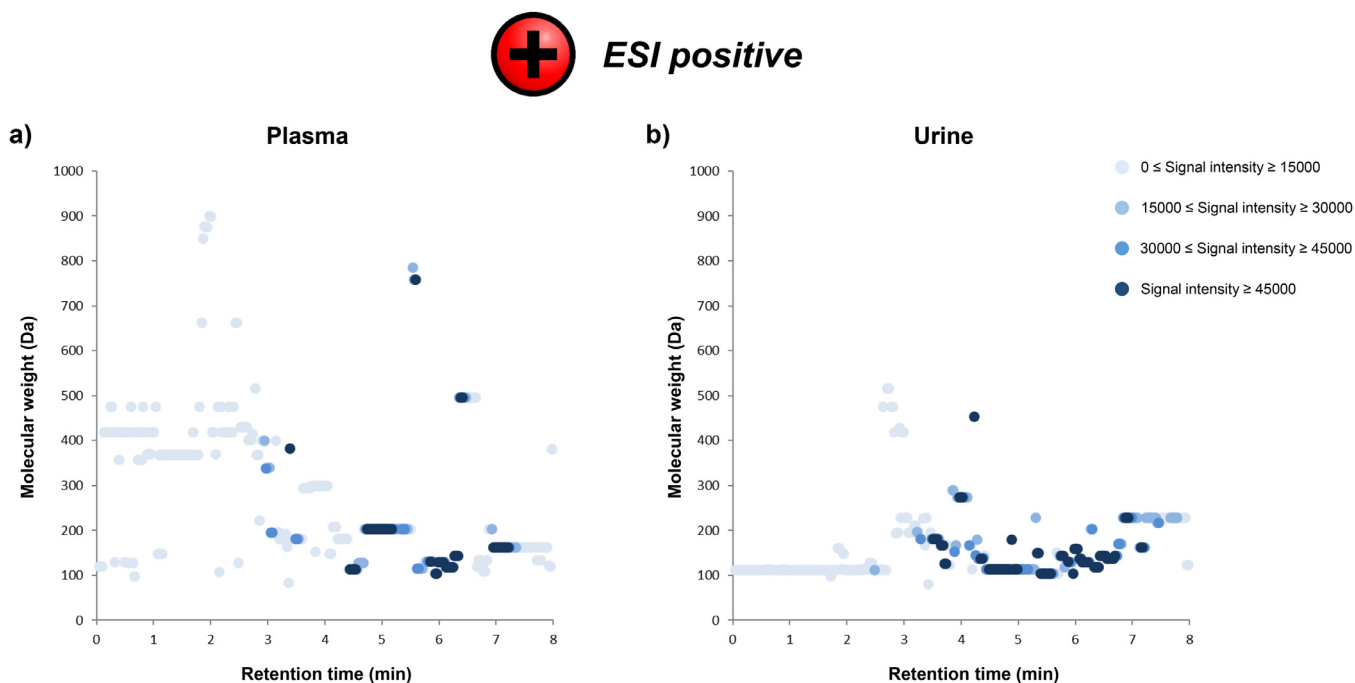

Fig. 2. (A) Ion map showing each compound belonging to the biological matrix assessed (plasma), according to their molecular weight (Da) and retention time (min). The signals with a more intense colour represent a higher signal intensity. (B) Ion map showing each compound belonging to the biological 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.
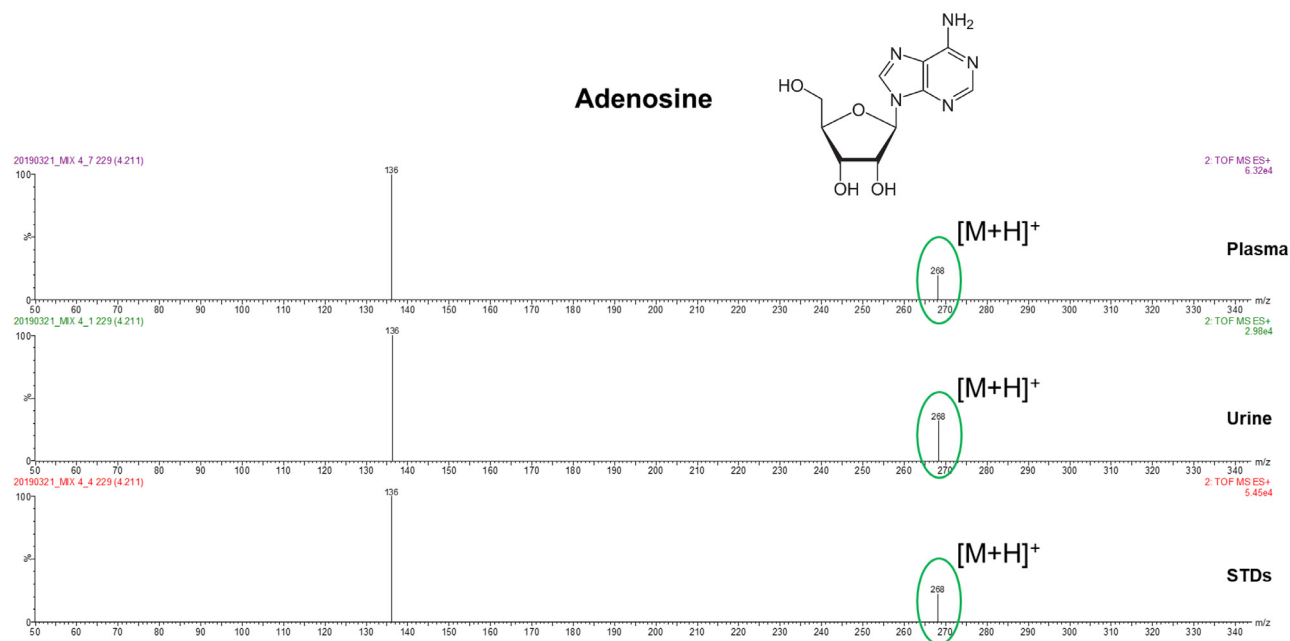
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Fig. 3. MS/MS spectra for adenosine in plasma (upper signal), urine (middle signal) and in neat standard solution (lower signal).

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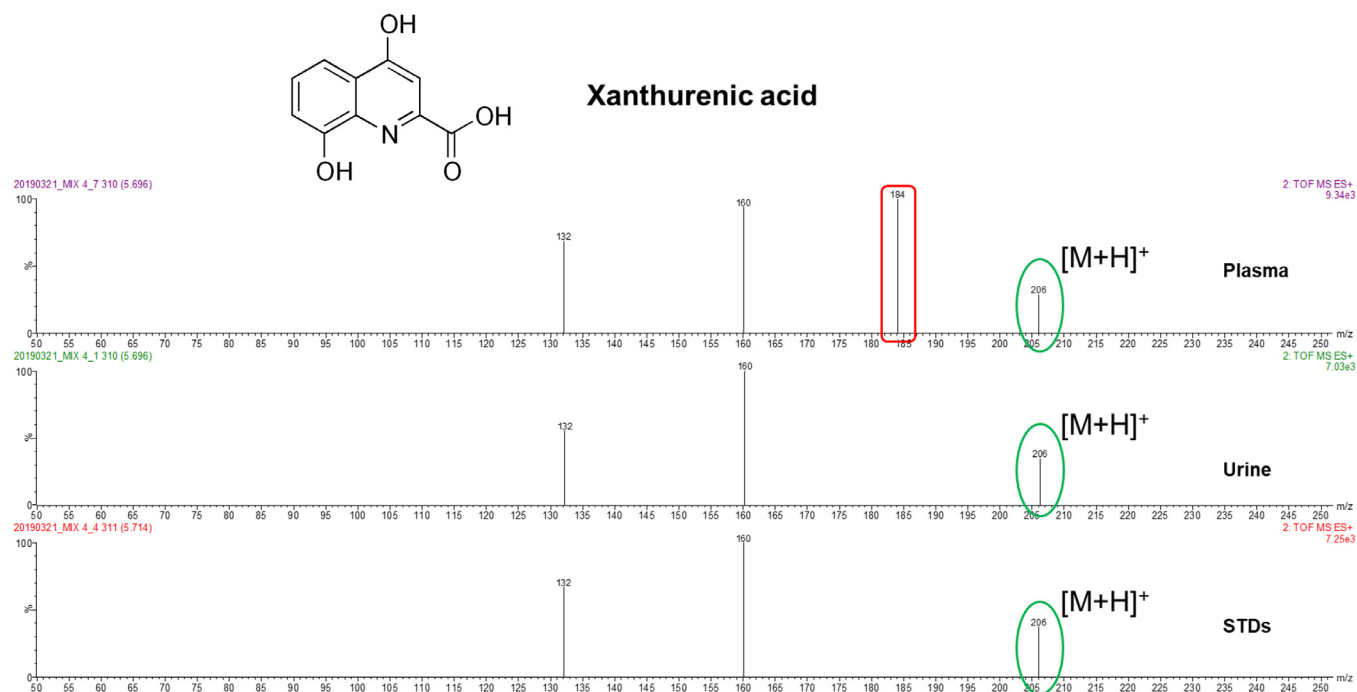
Fig. 4. MS/MS spectra for xanthurenic acid in plasma (upper signal), urine (middle signal) and in neat standard solution (lower signal).
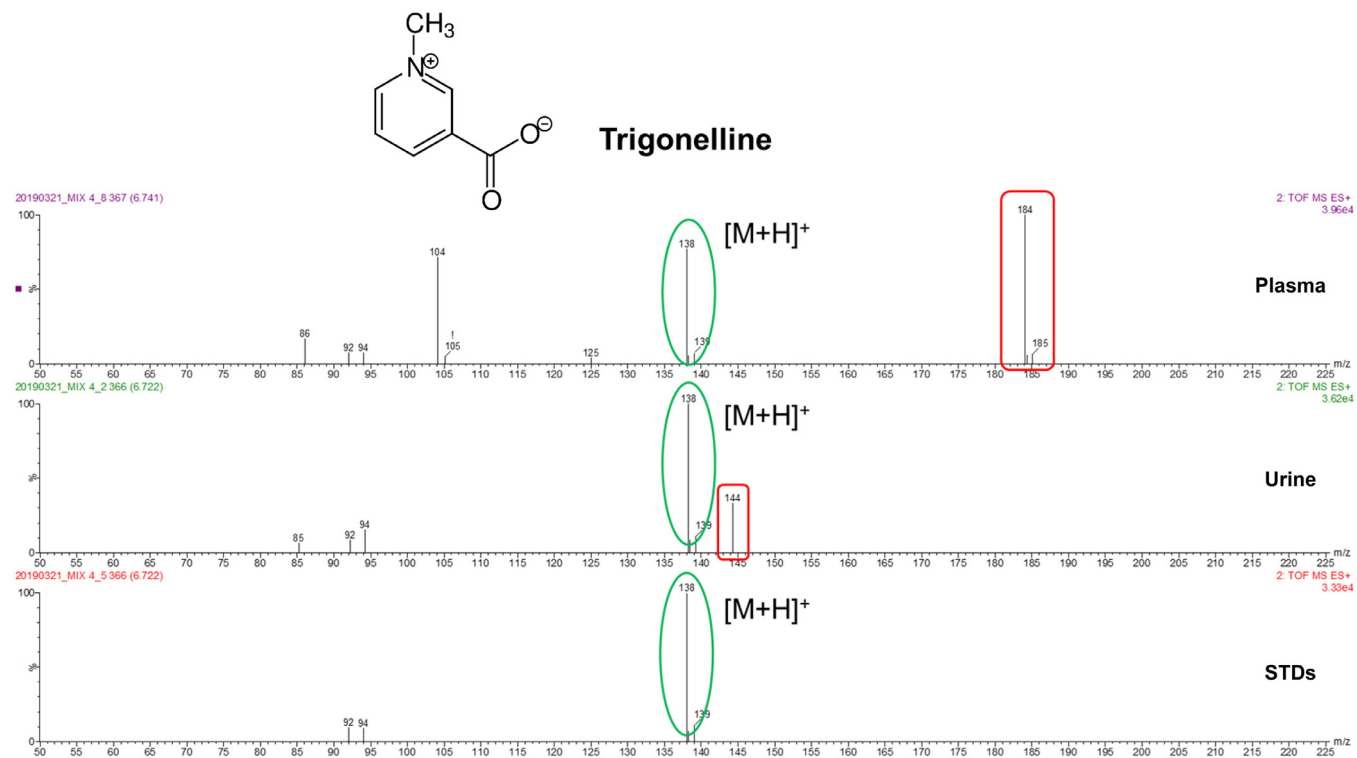

Fig. 5. MS/MS spectra for trigonelline in plasma (upper signal), urine (middle signal) and in neat standard solution (lower signal).

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 mod-

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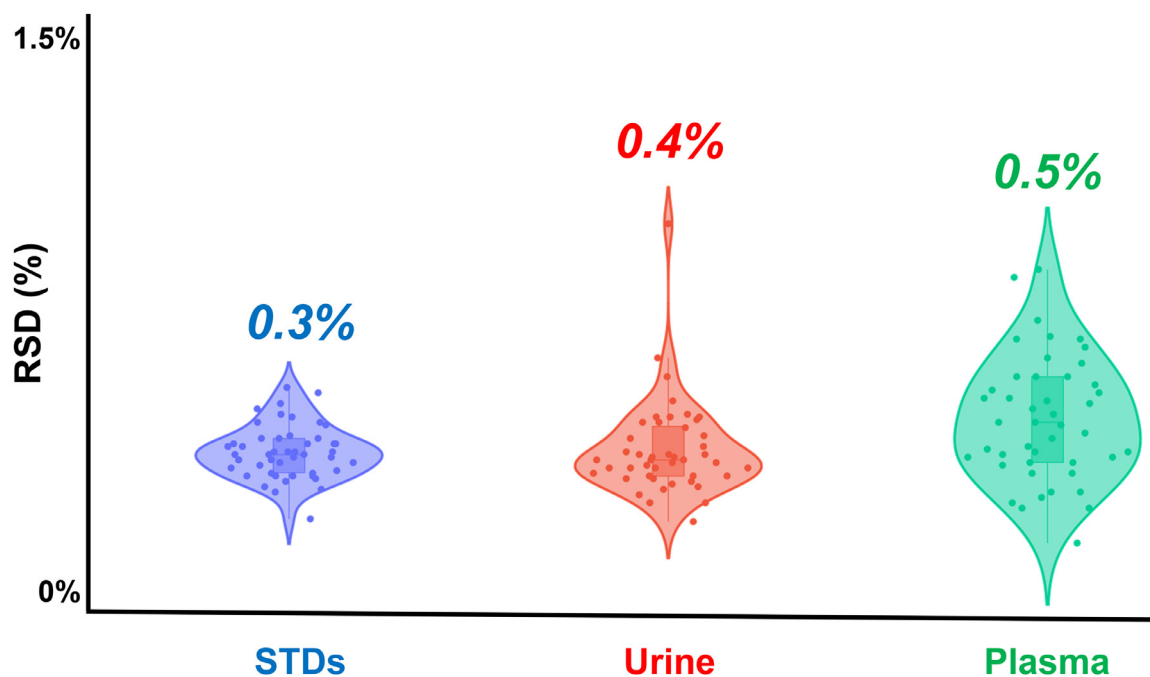
Fig. 6. Violin plots representing the population of RSD (%) values calculated for the 49 metabolites in neat standard solutions (blue), urine (red) and plasma (green). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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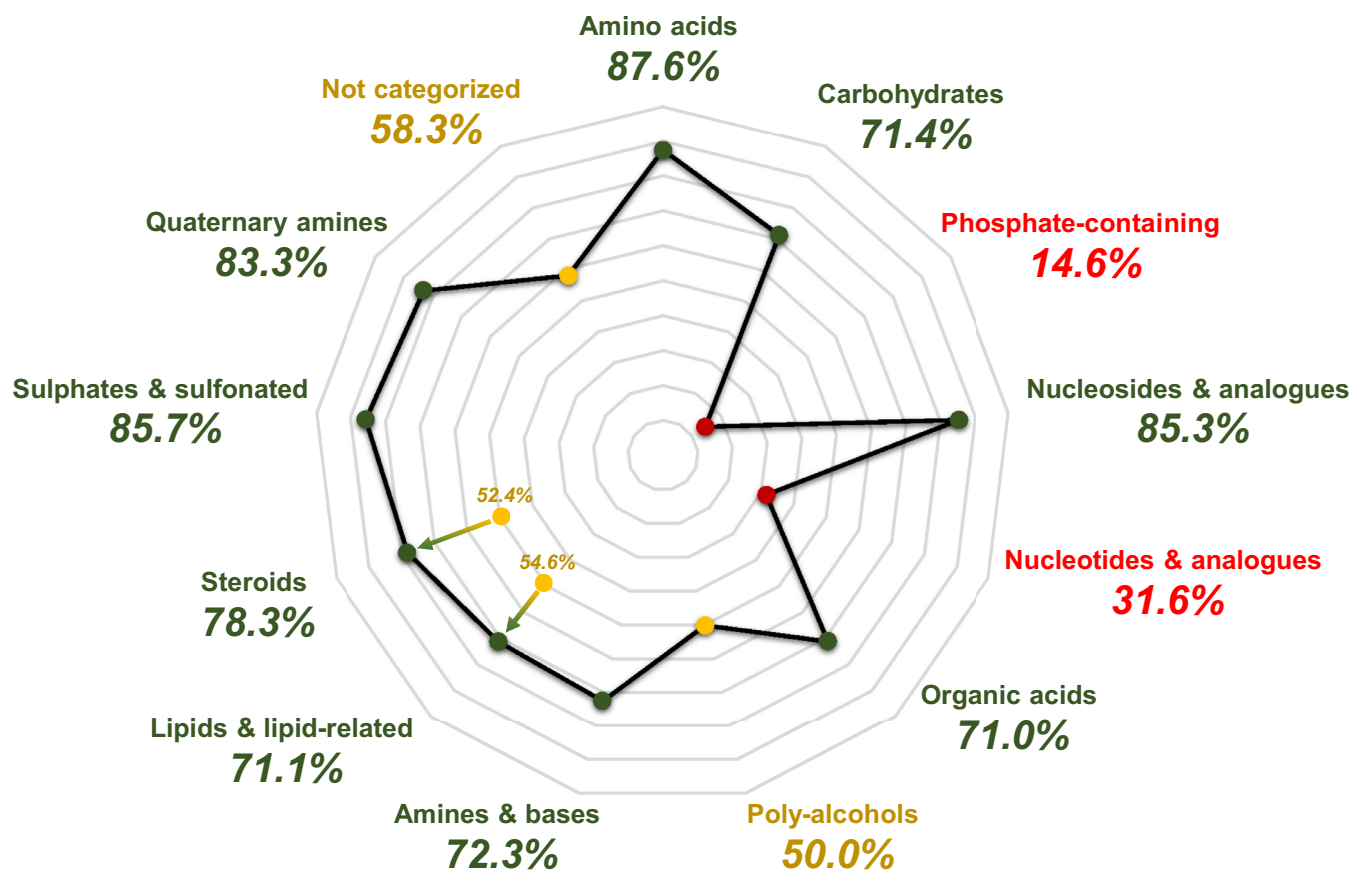
Fig. 7. Spider graph depicting the detectability percentages of each class of metabolites present in the Sigma MSMLS.
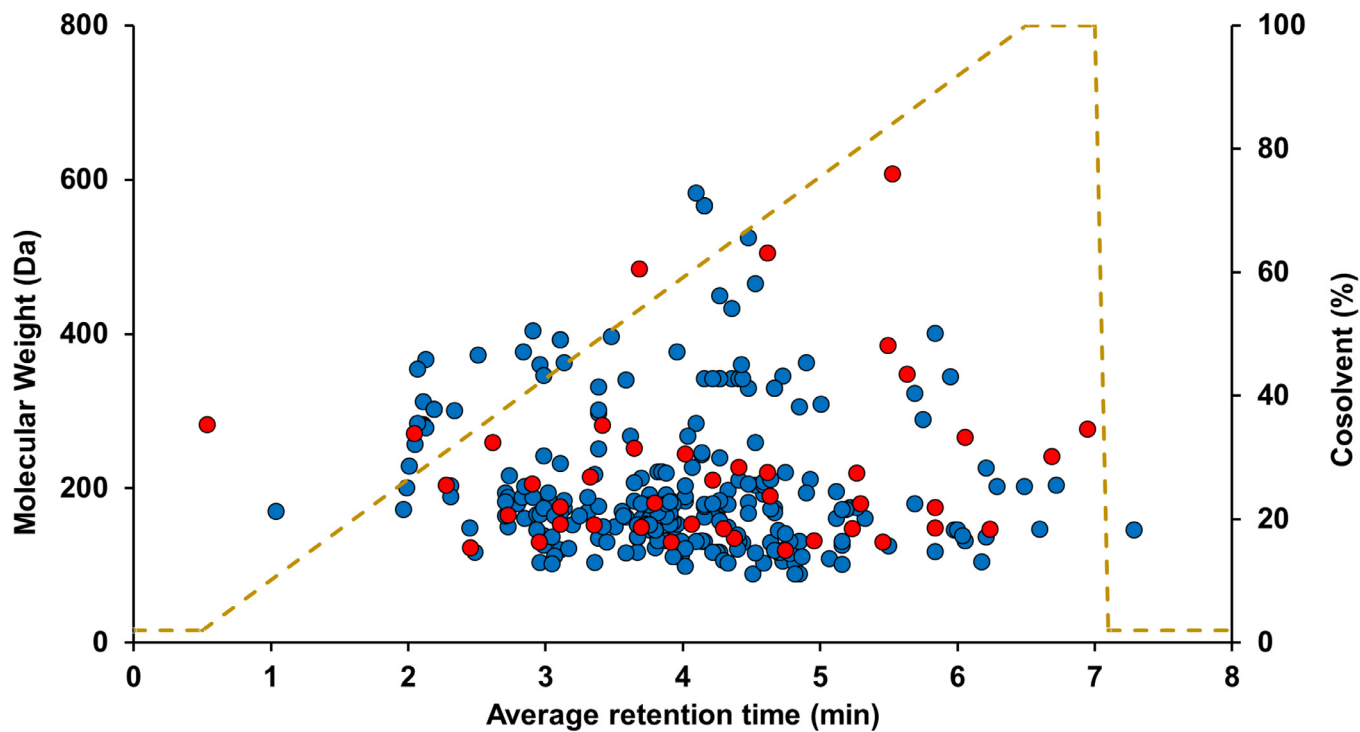

Fig. 8. Scatter plot of each detected metabolite from the Sigma MSMLS, according to their average retention times (min), molecular weights (Da) and percentage of cosolvent needed for their elution.

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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