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Supercritical Fluid Chromatography - Mass Spectrometry using Data Independent Acquisition for the Analysis of Polar Metabolites in Human Urine

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Highlights

- SFC shows complementarity to RPLC and HILIC for metabolomics.
- In SFC–MS the ESI response of metabolites can be tuned addition of make-up.
- SFC–MS analysis of urine metabolites using data independent acquisition (SWATH/MS).

Abstract

The application of supercritical fluid chromatograph with mass spectrometric (MS) detection (SFC-MS) was compared towards generic reversed phase liquid chromatography (RPLC) and hydrophilic interaction liquid chromatography (HILIC) for the analysis of urine with regards of ionization performance and analyte identification. The different chromatographic conditions were characterized with a selected set of 51 metabolites from different classes reported in the Human Metabolome DataBase (HMDB) and previously detected in human urine and/or plasma. SFC using a diol column with a gradient of carbon dioxide (CO_2) and methanol with 10 mM ammonium hydroxide as modifier was able to retain and separate twenty polar analytes co-eluting in the RPLC eluent front. In the conditions investigated and compared to HILIC where many metabolites were also co-eluting, SFC showed a different ratio between elution domain and analysis time. Similar peak width and symmetry were observed, while retention time variability was slightly lower compared to that of HILIC (0.15% versus 0.24% and 1.26% for RPLC and HILIC, respectively). In SFC-MS, a significant signal enhancement (2-150 times, average of about 10 times) was measured after post-column make-up addition ($MeOH/H_2O$, 95/5, v/v + 25 mM ammonium acetate) for twenty-eight analytes. Nine analytes measured by LC-MS could not be detected in SFC-MS. Applicability of SFC-MS for metabolomics was investigated with the analysis of urine samples using data independent acquisition (DIA) and more specifically Sequential Window Acquisition of all Theoretical Mass Spectra (SWATH/MS). Using a metabolomics library, 74 metabolites from human urine could be identified in positive mode in a single SFC-MS analysis of 15 minutes.

1. Introduction

The current challenge in metabolomics is to identify and quantify more informative disease biomarkers, aid the design or development of improved treatments, and better assess health outcomes. These actions are vital tools for drug discovery and therapeutics [1]. For that purpose, metabolomics requires the use of both analytical chemistry and advanced computational methods [2]. 114'100 metabolites are reported in the current version of the Human Metabolome DataBase (HMDB) (version 4.0)[3], among which there are around 20% of metabolites (23'746) including polar metabolites such as acids, amino acids, nucleotides or sugars and a large majority of lipids (90'354). These two main classes differ according to their chemical space (molecular weight, polarity, pKa, concentration dynamic range, MS response). Compared to lipids, polar metabolites present a wider chemical diversity especially regarding their structural features, polarity range and their pKa, which make them more challenging to be analyzed with a single assay. The aim would be to analyze and quantify as many metabolites as possible, combining high-throughput analysis, large metabolite coverage, automation, accurate quantitation and low-cost analysis. Mass Spectrometry (MS) offers best for sensitivity and selectivity, and it can be hyphenated in a straightforward manner with separation techniques [1] such as Gas Chromatography (GC), Liquid Chromatography (LC), Supercritical Fluid Chromatography (SFC) or capillary electrophoresis (CE).

Supercritical Fluid Chromatography (SFC) was described for the first time in 1962 [4] and became an alternative to normal phase chromatography or gas phase chromatography, using either packed or open-tubular columns. In the last decade, SFC using packed columns has experienced a renaissance thanks to the commercialization of new hardware [5] also in combination with mass spectrometric detection. SFC started to be applied in metabolomics mostly for apolar compounds. Therefore since the beginning of the 2000's, many lipidomics studies were carried out using SFC-MS to perform lipid profiling of various matrices (leaf extract [6], soybean [7], sheep [8] and mouse [9] plasma or dried plasma spot [10]). Lisa *et al.* carried out analysis of porcine brain extracts, and were able to separate lipids into 30 lipid classes in 6 minutes [11].

The availability of column chemistries from polar (diol, silica) to apolar (C18) and the use of modifiers such as methanol, or additives such as ammonium acetate [12], have extended the applications of SFC, becoming a real alternative to liquid chromatography [13]. More recently SFC was applied for the analysis of polar metabolites. Among these studies, Plumb *et al.* [14] performed SFC analysis for metabolic profiling of rat and dog bile, using a gradient from 1 to 10% of methanol (MeOH). They were able to do untargeted analysis of more than 100 bile samples with robustness and performance comparable to Reversed-Phase Liquid Chromatography (RPLC), reducing both overall organic solvent consumption and analysis time. Sen *et al.* determined optimal conditions for the analysis of polar compounds and showed the importance of adding polar additives such as ammonium salts in order to improve chromatographic performances. Sixty polar urinary metabolites could be identified [15]. Recently in 2018, Desfontaine *et al.* [16] investigated the applicability of SFC-MS for the analysis of a

wide range of hydrophilic and lipophilic analytes in a single analysis, using a gradient starting from 2 to 100% modifier consisting of a mixture of 5% water (H_2O) in MeOH, with 50 mM of ammonium formate (NH_4FA) and 1 mM of ammonium fluoride.

The interfacing of SFC to MS is straightforward but some differences take place when compared to LC-MS, especially the presence of a back pressure regulator (BPR) and a post column make-up pump. The BPR serves to maintain the pressure in the column, and the role of the make-up pump is to prevent analyte precipitation during the CO_2 decompression after the BPR. In LC-MS, the electrospray ionization conditions are predominantly determined by the mobile phase composition, which considerably varies in gradient mode. The use of a make-up in LC-MS post column would result in a dilution effect affecting the MS response in electrospray which behaves like a concentration-sensitive detector [17]. In SFC, the make-up can be used to enhance the ionization by tuning its composition and improves limit of quantification [18].

Selected Reaction Monitoring (SRM)/MS is widely used for routine quantitative analysis due to its high sensitivity and robustness. However, only metabolites for which SRM transitions are known can be measured and transitions needs to be optimized prior to analysis. High resolution mass spectrometry has gained in interest over the years for quantitative analysis. In this context, data independent acquisition techniques (DIA) such as SWATH (Sequential Window Acquisition of all Theoretical Mass Spectra) have emerged during the last decade [19]. Contrary to data dependent acquisition (DDA), where only the ions above a determined threshold from the first quadrupole (Q1) are selected for fragmentation, in DIA, all ions going through Q1 are fragmented generating a comprehensive data set. This allows improved coverage and reduced method development time for qualitative or quantitative analysis as the data can be interrogated at any time.

The present work investigates the potential of SFC-MS for the qualitative and quantitative analysis of polar metabolites in biological matrices, and in particular urine, with respect to analysis time, chromatographic resolution and MS response. The resolution of SFC separation is compared to other commonly used liquid chromatographic methods (RPLC and HILIC) using a representative set of metabolites. The effect of the post-column make-up addition on the ionization performance is also evaluated. Finally, the benefits of SFC with SWATH/MS regarding identification/quantification of metabolites in human urine samples with the help of MS/MS library are explored.

2. Materials & methods

2.1 Chemical and reagents

MeOH, and isopropanol (2-PrOH), HPLC grades, were purchased from Fischer Scientific (Loughborough, UK), acetonitrile (CH_3CN) was purchased from Biosolve B.V. (Valkenswaard, Netherlands). Pressurized liquid carbon dioxide (CO_2), 3.0 grade, (99.9%) was purchased from PanGas (Dagmerstellen, Switzerland). Formic acid (FA) (>98%) was provided from Merck Millipore

Corporation (Darmstadt, Germany), ammonium hydroxide (NH_4OH) (25% in MeOH) from VWR (Fontenay-sous-Bois, France), NH_4FA and ammonium acetate (NH_4Ac) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The metabolites (carnosine, *N*-acetyl-L-phenylalanine, L-lysine, homocitrulline, betaine, L-glutamine, creatine, homo-L-arginine, L-proline, L-histidine, L-aspartyl-L-phenylalanine, hippuric acid, 1-methylhistidine, quinaldic acid, phenylacetylglycine, 4-guanidinobutanoic acid, isovalerylglycine, ethenodeoxyadenosine, cyclic AMP, guanosine, 5'-methylthioadenosine, 1-methyladenosine, 3-methyladenine, indoleacetic acid, tyramine, urocanic acid, riboflavin, 1,3-dimethyluric acid, acetaminophen, chlorogenic acid, N-methylnicotinamide, 7-methylguanine, 1,3,7-trimethyluric acid, theobromine, phloretin, 1,9-dimethyluric acid, L-carnitine, creatinine, pantothenic acid, diethanolamine, azelaic acid, N-acetylputrescine, glycocholic acid, cortisone, taurocholic acid, glycerophosphocholine, L-acetylcarnitine, monomethyl glutaric acid, trigonelline, cotinine, N-acetylneuraminic acid) were purchased from Sigma-Aldrich (St. Louis, MO, USA). This mix of 51 analytes is representative of metabolites already detected in urine and/or plasma [20, 21] and covers a wide range of physico-chemical properties, representative of the chemical diversity of the polar metabolites constituting of the HMDB (molecular weight from around 100 to more than 500 g/mol, log P from around -6 to 3 and pKa from around -3 to 16), and different metabolite classes such as amino acids and peptides, nucleosides and nucleotides, aromatic heteromonocyclic compounds, aliphatic compounds, organic acids, steroids and derivatives, lipids, alkaloids and carbohydrates and conjugates are represented. Physicochemical properties, SRM transitions and structures are presented in Electronic Supplementary Material (ESM), Table S1.

A pool of human urine was made using six anonymous donors and was stored at -20°C prior analysis.

2.2 Analytical conditions

2.2.1 SFC analysis

SFC analysis were carried out on the Nexera UC system (Shimadzu Corporation, Kyoto, Japan) equipped with a CBM-20A controller module, a LC-30ADSF CO_2 pump, a LC-20ADXR modifier pump, a LC-30AD make-up pump, a SIL-30AC auto sampler, a CTO-20AC oven and a SFC-30A Back Pressure Regulator.

The analytes were separated on Kromasil diol stationary phase (AkzoNobel, 3.1 x 150 mm, 2.5 µm) with a mobile phase constituted of CO_2 and MeOH with 20 mM of NH_4OH as mobile phase additive. Analyses were carried out in gradient mode from 5 to 50 % of modifier in 10 minutes, at a flow-rate of 1.5 mL/min. The pressure and temperature conditions were 150 bars at 40°C. The make-up flow-rate was set at 0.3 mL/min and consisted of a mixture of MeOH/H_2O, 95/5, v/v with 25 mM of NH_4Ac[18].

2.2.2 LC analysis

In LC, analyses were carried out on a HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with a CBM-20A$_{lite}$ controller module, a quaternary pump LC-30AD with low pressure gradient mode, a SIL-30AC auto sampler and a CTO-30A oven.

Published separation conditions were used to compare generic RPLC[20] and HILIC[22] methods with SFC separation. RPLC separations were given by a X select HSS T3 XP (Waters, 2.1 x 150mm, 2.5 µm) at 40°C, with a mobile phase constituted of A = H_2O + 5 mM NH_4FA, pH = 3 with FA and B = MeOH in positive mode and A = H_2O + 5 mM NH_4Ac, pH = 8 with NH_4OH and B = MeOH in negative mode, at a flow-rate of 0.3 mL/min with the following gradient 0-1 min: 5% B, 1-20 min: 5 to 95% B, 20-25 min:95% B, 25-28 min: 5% B. HILIC separations were given by a X Bridge Amide (Waters, 2.1 x 100mm, 3.5 µm) at 40°C, with a mobile phase constituted of A = H_2O/CH_3CN, 95/5, v/v + 20 mM NH_4OH + 20 mM NH_4Ac and B = CH_3CN, at a flow-rate of 0.3 mL/min with the following gradient 0-3 min: 85 to 30% B, 3-12 min: 30 to 2% B, 12-17 min: 85% B.

2.2.3 MS conditions

For SRM analysis, the different chromatographic systems were coupled to a triple quadrupole mass spectrometer (QqQ) LCMS-8050 (Shimadzu, Duisburg, Germany) through an electrospray ion source using positive and negative mode. MS conditions were as follows: capillary voltage = 4.5 kV, temperature of desolvation line = 250°C, temperature of heating block = 400°C, interface temperature = 300°C, nebulizing gas = 3 L/min (air), drying gas = 10 L/min (N_2), heating gas = 10 L/min, Collision Induced Dissociation (CID) gas = 17 kPa (argon). The dwell time was set at 10 ms for each transition. SRM transitions and collision energies (CE) were optimized with the LabSolutions software and are summarized in ESM, Table S1. The voltages at Q1 prebias, collision cell and Q3 pre bias were optimized for each analyte to obtain the highest sensitivity. LabSolutions software was used to control the chromatographic instruments and to collect the data from the LCMS-8050 QqQ [18].

For SWATH acquisition, a TripleTOF 5600 (AB Sciex, Concord, ON, Canada) was used. A single TOF MS acquisition was followed by 28 MS/MS experiments with Q1 windows of 25 units covering a mass range from *m/z* 100 to 800 for positive mode. The cycle time was adjusted to 852 ms based on an average LC peak width of 12 s, to obtain at least 12 points/ peak. For MS/MS a collision energy spread of 40 ± 30 eV was applied and fragments were recorded from m/z 50 to 800. The sprayer capillary voltage was of 5000 V, with a DP of ± 70 V and a source temperature of 400 °C. The curtain gas was set at 25 and the gas 1 and gas 2 at 30 and 50 respectively (laboratory frame).

PeakView 2.2 with the MasterView 1.1 package (Sciex) was used for data evaluation and library search of urine samples. The MasterView search criteria were set as follows: XIC intensities above 100 counts or S/N > 10, XIC width 10 mmu, with the following library search parameters: confirmation search, precursor mass tolerance of 0.4 Da (smaller tolerance could also be used), a fragment mass accuracy of 5 ppm, polarity filter was applied, an intensity threshold of 1%, a minimal purity of 0.1% and an intensity

factor of 100. An in-house made composite MS/MS spectra library (AMML) containing 532 metabolites represented in the human metabolite database (HMDB) was used for compound identification [21].

3. Results and discussion

3.1 Separation orthogonality: SFC versus RPLC versus HILIC

The RPLC and HILIC methods [20] [22] are generic methods which have been reported for metabolomics analysis. RPLC is mostly used for moderate to apolar metabolites while HILIC is applied to improve retention the polar metabolites and lipid class separation. On the other side SFC, using stationary phases with polar groups such as diol can cover a larger range of polarity complementary to RPLC and HILIC [23]. SFC resolution was compared with RPLC and HILIC, using a test mix of 51 metabolites detected in human urine and/or plasma and representative of the HMDB chemical diversity (ESM, Table S1). The metabolites a subset of 552 metabolites and representative for the chemical space with regard in RPLC separations. Out of 51 compounds 20 of them are known to be poorly retained on RPLC columns. In RPLC as expected, 20 analytes (polar metabolites with log P < 0) are co-eluting in the solvent front between 1.12 and 1.35 min (Figure 1A). The other 30 analytes are distributed over the 28 minutes gradient. The analysis of the test mix in HILIC brings a different selectivity (Figure 1B) with a stronger retention of polar metabolites but limited retention of the apolar ones. Most of the analytes are eluting around 2 and 3 minutes but in packets. The same behavior was reported by Asara *et al.* [22] for the analysis of 300 metabolites and illustrated limited chromatographic resolution in HILIC. Despite the fact that the method of Asara *et al.* is highly referenced, their conditions are sub-optimal and further improvements in chromatographic conditions (mobile phase, gradient, temperature) are possible. In SFC using a polar stationary phase (diol) compared to RPLC, higher retention is obtained for both polar and apolar metabolites (Figure 1C). Looking at three examples, namely glycerophosphocholine (log P = -5.88), *N*-acetyl-L-phenylalanine (log P = -0.04) and cotinine (log P = 0.17), a completely different selectivity is observed from one mode to another (ESM, Figure S1). This is also demonstrated comparing the resolutions between these analytes (ESM, Table S3). All three separation techniques show different analyte behaviors. Further optimization could be performed in particular for the HILIC conditions, but when dealing with a large analyte chemical space, method optimization is often on cost of time, sample throughput and generic use of the method. Moreover, with mass spectrometric detection, baseline separation of all analytes is not a must and can even be of interest.

The retention times of the different chromatographic modes were compared by pairs of separation methods for each analyte. The packet of 20 analytes co-eluting in RPLC can be separated by HILIC (Figure 2A). A similar observation can be made between RPLC and SFC (Figure 2B). Finally, the comparison between HILIC and SFC (Figure 2C) confirms that the SFC separation is not comparable to HILIC and brings an additional separation dimension. Regarding the chromatographic performances, namely peak width (ESM, Figure S2) and peak symmetry (ESM, Figure S3), the peak widths are

comparable for all of methods even if a few more metabolites show larger peak width in SFC (especially basic ones such as L-glutamine, L-lysine or L-proline) and the peak symmetry is similar. Regarding the retention time stability, HILIC shows much more variability compared to RPLC and SFC (1.26% on average versus 0.24 and 0.15% for RPLC and SFC, respectively) (ESM, Figure S4). The retention time variability can certainly be improved for HILIC with longer equilibration time and optimized conditions but on cost of sample throughput and assay flexibility.

3.2 Ionization factor enhancement

SFC has been applied for the analysis of polar and apolar analytes but with different separation conditions. Generic separation for a large range of analytes [16] is highly desirable but the absolute concentrations and concentration ranges of all compounds in biological samples, regarding sample volume, have to be taken into account. The ionization might be unified in order to have the possibility to separate and detect the analytes in a single run. The MS signal intensities using ESI in positive and negative mode are compared for RPLC or SFC separations. Figure 3 shows the MS response for 6 metabolites in LC-MS and SFC-MS using the post-column make-up addition of MeOH/H_2O, 95/5, v/v with 25 mM of NH_4Ac at 0.3mL/min. The observed enhancement in SFC-MS for metabolites is certainly due to the addition of NH_4Ac in the make-up, as already described previously for pharmaceuticals and low molecular weight compounds [18]. Figure 4 plots the enhancement factor in SFC-MS versus LC-MS for the mix of 51 analytes. The red line indicates a ratio of 1 which means that LC-MS and SFC-MS signals are equal. Above the red line, the SFC-MS signal is higher, whereas below it, it is the LC-MS signal which is higher. For a large part of the analytes (28/51), a significant enhancement is observed for SFC-MS, while almost 16% of analytes (8/51) provide better signal in RPLC-MS. Similar results are observed in negative mode (ESM, Figure S5). Figure 5 describes the repartition of the enhancement factors of MS signal intensity for positive mode (Figure 5A) and negative mode (Figure 5B) using SRM/MS acquisitions. Compared to LC-MS, in SFC-MS about 24 % and 32 % fewer analytes can be detected in positive or in negative mode for the mix of 51. These analytes not seen in SFC-MS as they apparently did not elute from the SFC column in the conditions investigated. On the other hand, 18 % of the analytes can only be detected in SFC-MS in negative mode. This is certainly due to the better ionization conditions in SFC-MS versus LC-MS or to the different detection limits depending on the separation conditions. Regarding MS signal enhancement, above a factor of ten was only observed for about 10 % of the analytes. The values are detailed in ESM, Table S4. To conclude, neither RPLC nor SFC provided different enough separation selectivity to separate all 51 metabolites, while the largest difference between the two separation modes was observed with enhanced MS ionization in SFC-MS, which is due to the controlled make-up flow conditions. No correlation was found between retention time and ionization enhancement factor, meaning that the enhancement is not related to the percentage of modifier at the elution but mostly to the make-up addition. No correlation could be established either regarding the enhancement factors in SFC-MS versus LC-MS considering retention time, pKa or log(D).

The MS signal responses were investigated considering the SRM transitions starting with protonated or deprotonated precursors. One cannot exclude that adducts distribution in the different investigated conditions may be different and affects analyte detection [24].

3.3 SWATH/MS acquisition of human urine samples for qualitative and quantitative analysis

LC-SRM/MS is widely used for sensitive and accurate quantification but requires defining the transitions of the analytes prior to analysis. High-resolution mass spectrometry as gained of interest for quantitative analysis but mostly in the selected ion monitoring mode using narrow windows (HR-SIM). Contrary to DDA where no quantification can be performed in MS2 mode over the LC peak, DIA such as SWATH allows to perform identification/characterization and quantification of almost any ionized analyte (QUAL/QUANT). A typical SWATH acquisition consists of a full scan experiment followed by 28 MS/MS experiments with Q1 windows of 25 units with a mass range from *m/z* 100 to 800 for positive mode for total cycle time of about 800 msec. For qualitative analysis, two approaches are possible based on HRMS: i) MS1 mode with peak detection, elemental formulae, isotopic ratio followed by chemical database search and confirmation by retention with an authentic standard or ii) MS1 mode with peak detection, elemental formulae, isotopic ratio followed by MS/MS libraries search. In the present investigation, the second approach was applied, as described previously by Bruderer *et al.* [21] using reversed phase chromatography with reference compounds and an in-house MS/MS library containing 532 metabolites represented in HMDB. Figure 6A shows the extracted ion current of 74 metabolites (ESM, Table S5) identified in SFC-SWATH/MS analysis of a human urine sample based on the match of the elemental formulae, isotopic ratio, library spectra and retention time. Compared to RPLC, where creatinine and creatine mostly co-elute, they could be nicely separated with SFC. This is of importance as creatine ($[M+H]^+$, *m/z* 133) can generate an ion at *m/z* 115 by up-front CID in the interface which is isomeric to the precursor ion of creatinine. To notice is the good match of the library spectra and the experimental spectra (Figure 6B-6D). The same urine sample was analyzed by SFC, RPLC and HILIC with SWATH/MS acquisition. A metabolite was considered as identified if a positive match was shown for at least 80% of repeated analysis (n=5). In total, 91 different metabolites were identified with all the techniques (ESM, Figure S6 and Table S6). 35.2% of the identifications were common for all chromatographic modes, whereas 37.4% were seen with one chromatographic separation mode only (23.1, 11.0 and 3.3% for RPLC, HILIC and SFC respectively). The extracted ion current profile of the common identified metabolites (n=41) is illustrated in Figure 7. In RPLC, all the analytes are eluted before 10 minutes on a run of 28 minutes (including re-equilibration) and there is a co-elution of the 18 most polar metabolites at the beginning of the gradient (Figure 7A). In HILIC, despite an orthogonality regarding the selectivity compared to RPLC separation, the analytes are eluted in a packet in less than 5 minutes on a run of 23 minutes (including re-equilibration) (Figure 7B). Furthermore, HILIC showed the largest retention time variability (ESM, Figure S4) under the investigated conditions. For both RPLC and HILIC, the ratio between elution domain and analysis time is very low, whereas in SFC, the

identified analytes are eluted between 2.7 and 9.2 minutes on a gradient of 15 minutes (including re-equilibration) (Figure 7C). The retention time's separation is more spread out with SFC analysis, as reported previously for polar metabolites[14] [15].

Selectivity of MS1 quantification depends largely on instrument resolution. It was previously shown that HR-SRM, using a resolving power of about 30'000, improves the signal to noise compared to HR-SIM [25] and therefore the limit of quantification. The signal-to-noise has been measured using both MS1 and MS2 for the representative analytes creatinine, urocanic acid and creatine and an increase in a range of 2 to 3 times was observed for MS2.

4. Conclusions

No single chromatographic system can cover the chemical diversity encountered in metabolomics. HILIC is largely used for the analysis of polar metabolites with the benefit of high organic mobile phase for enhancing ESI-MS response but suffers from relatively long re-equilibration times. RPLC simply does not retain these metabolites and early eluting metabolites are strongly affected by matrix effects in electrospray ionization. To point out is that with hyphenation of separation technique to mass spectrometry, not only the separation technique has to be considered, but also the ionization conditions and the MS acquisition mode. Ideally, one would like to use multiple chromatographic systems but on cost of analysis time and data processing. The major benefits of SFC are the possibility to identify and quantify metabolites in biological samples in less than 15 minutes with improved chromatographic resolution and the possibility to decouple separation from ESI conditions. As CO_2 expands prior MS detection and thanks to the use of a make-up, the chromatographic conditions can be optimized independently from the ionization conditions and does not need any adaptation of the electrospray source. The practicality of SFC-MS for metabolomics was demonstrated with the analysis of human urine samples where 74 metabolites could be detected in a single analysis. The combination of SFC with high-resolution mass spectrometry becomes particularly attractive with DIA such as SWATH/MS as it enables to obtain qualitative and quantitative information in a single analysis at the MS2 level. With SWATH/MS, the sample can be re-interrogated at any time. Metabolite libraries are useful to identify analytes in an automated way but it allows also building quantitative methods, post-acquisition, using HR-SRM, which is more selective than HR-SIM. When ultimate sensitivity is required, these methods could be easily transferred to triple quadrupole instruments.

5. Compliance with ethical standards

The human urine samples were anonymously collected. The Human Research Act (HRA) does not apply for the anonymized urine sample analyzed in the present work (Art. 2 para. 2 let. b and c).

6. Conflict of interest

The authors declare that they have no conflict of interest.

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8. List of Figures

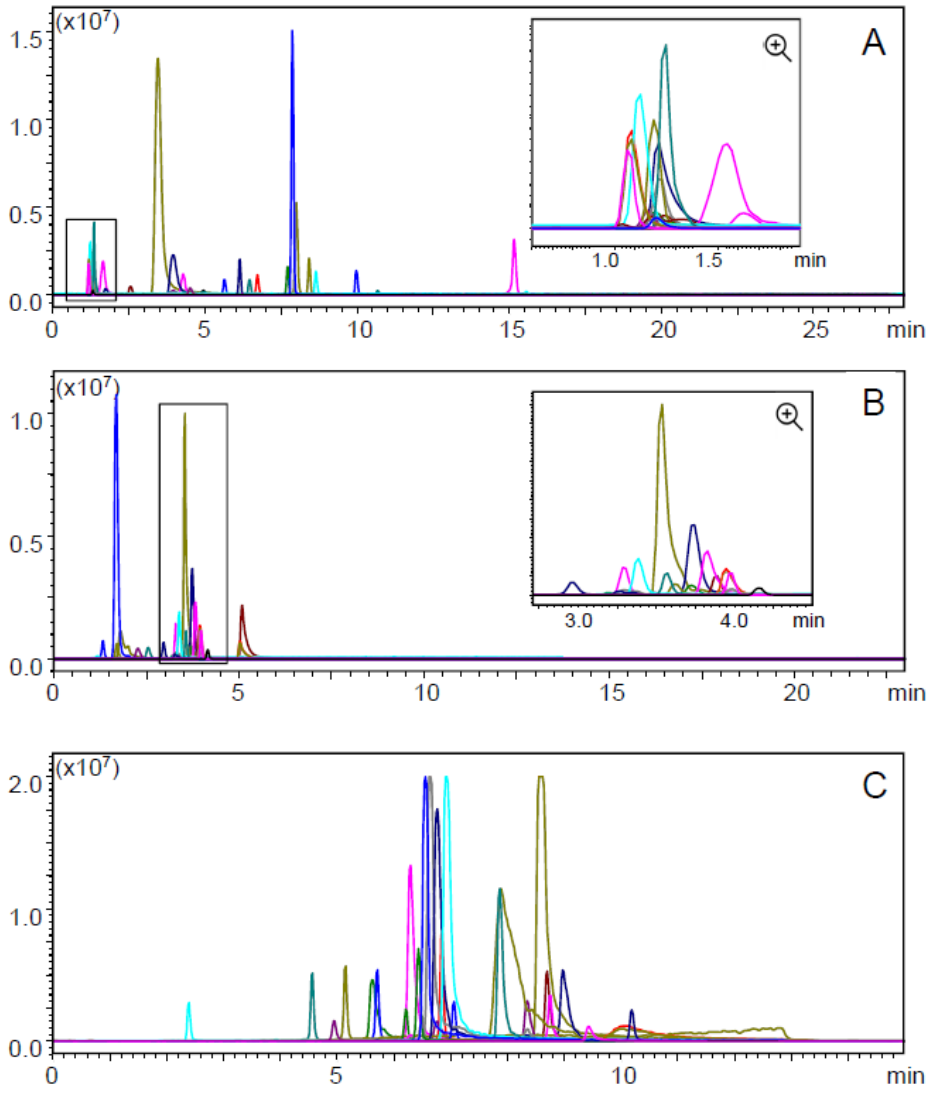

Figure 1. Chromatographic separation of the mix of 51 metabolites in RPLC A), HILIC B) and SFC C); for analyte assignment see ESM, Table S2.

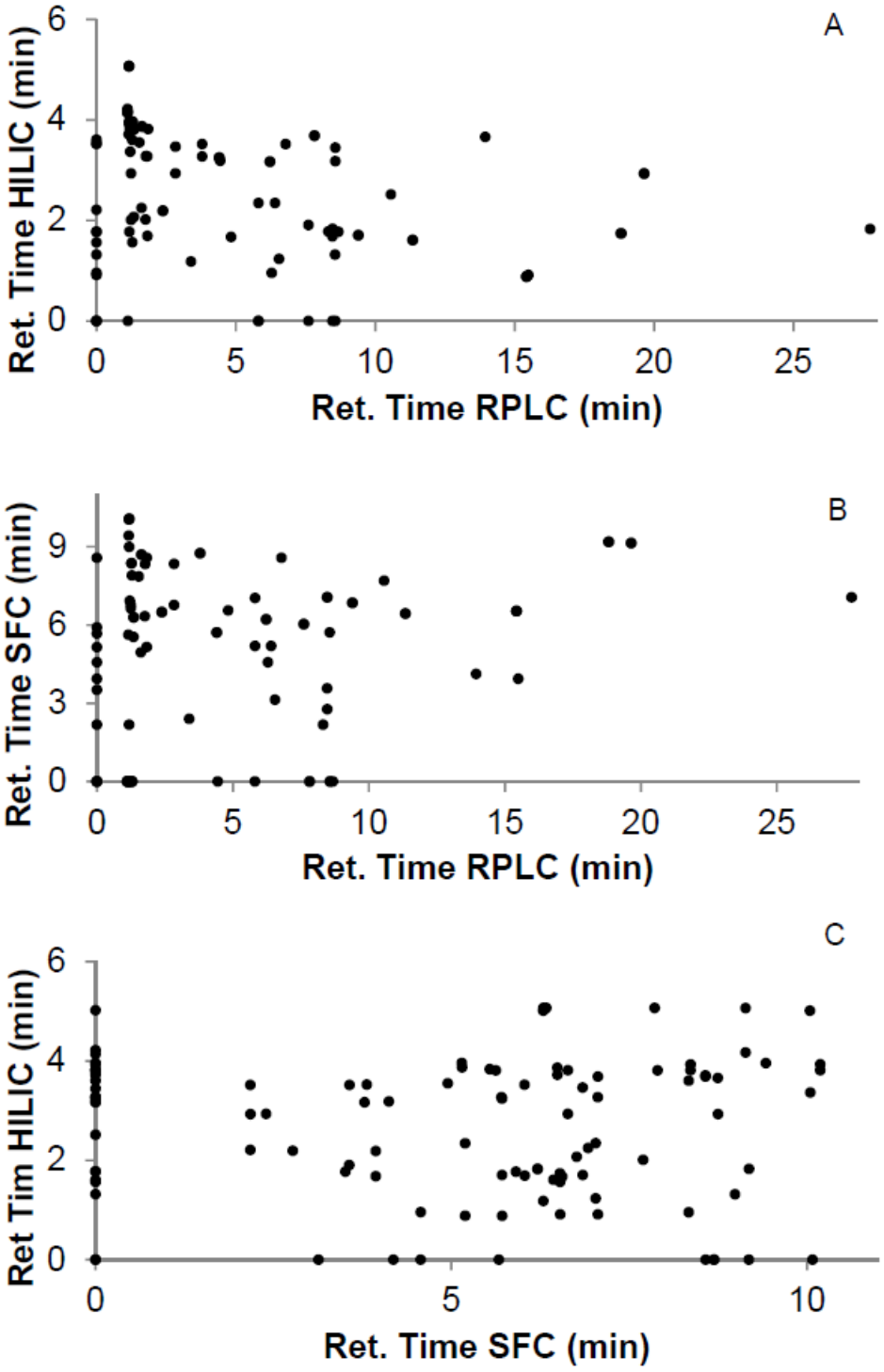

Figure 2. Pair comparisons of chromatographic modes regarding retention times, A: RT HILIC = f(RT RPLC); B: RT SFC = f(RT RPLC); C: RT HILIC = f(RT SFC).

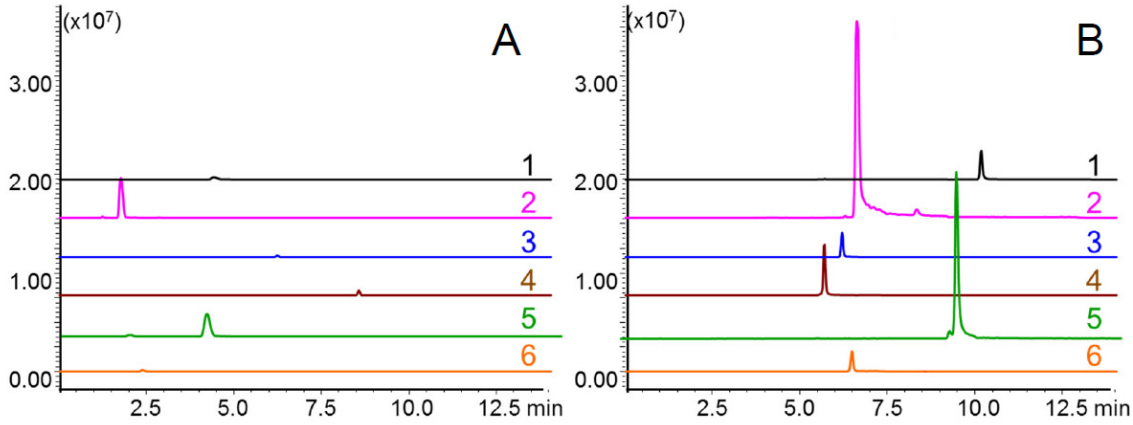

Figure 3. Comparison of RPLC-MS (A) and SFC-MS (B) chromatograms, showing the MS signal intensity increase in SFC-MS with the post-column make-up addition of MeOH/H_2O (95/5, v/v) + 25 mM NH_4Ac (1: cyclic AMP 2: urocanic acid 3: pantothenic acid 4: 5'methylthioadenosine 5: guanosine 6: 7-methylguanine).

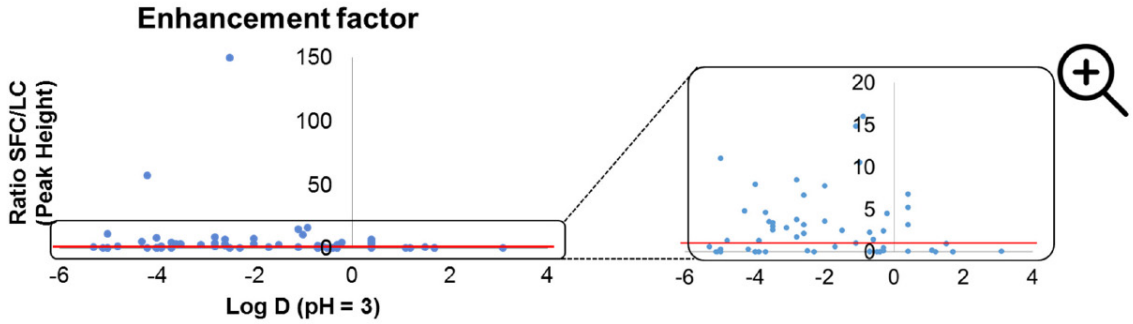

Figure 4. Enhancement factors observed in SFC-MS when plotting the peak height ratio of SFC-MS/LC-MS in positive mode. The red line represents a ratio of 1.

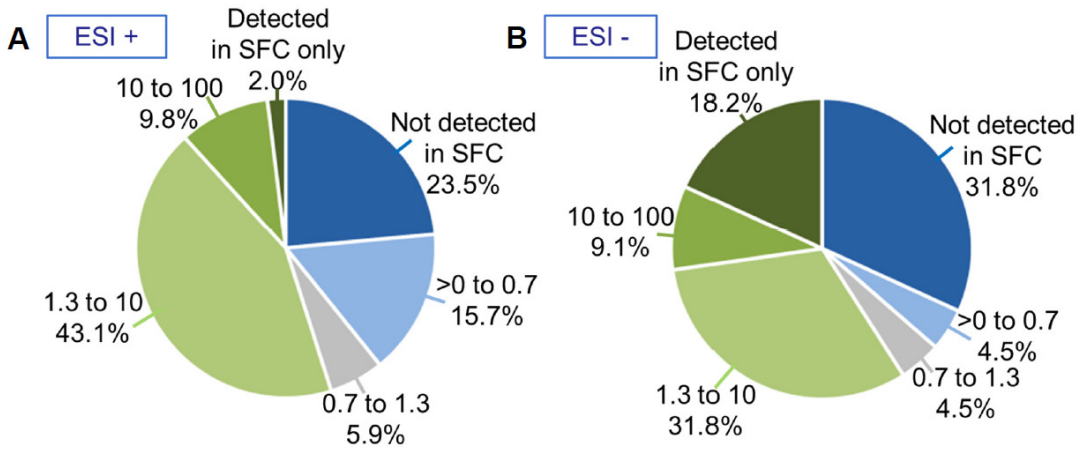

Figure 5. Distribution of the enhancement factors in positive (A) and negative (B) modes.

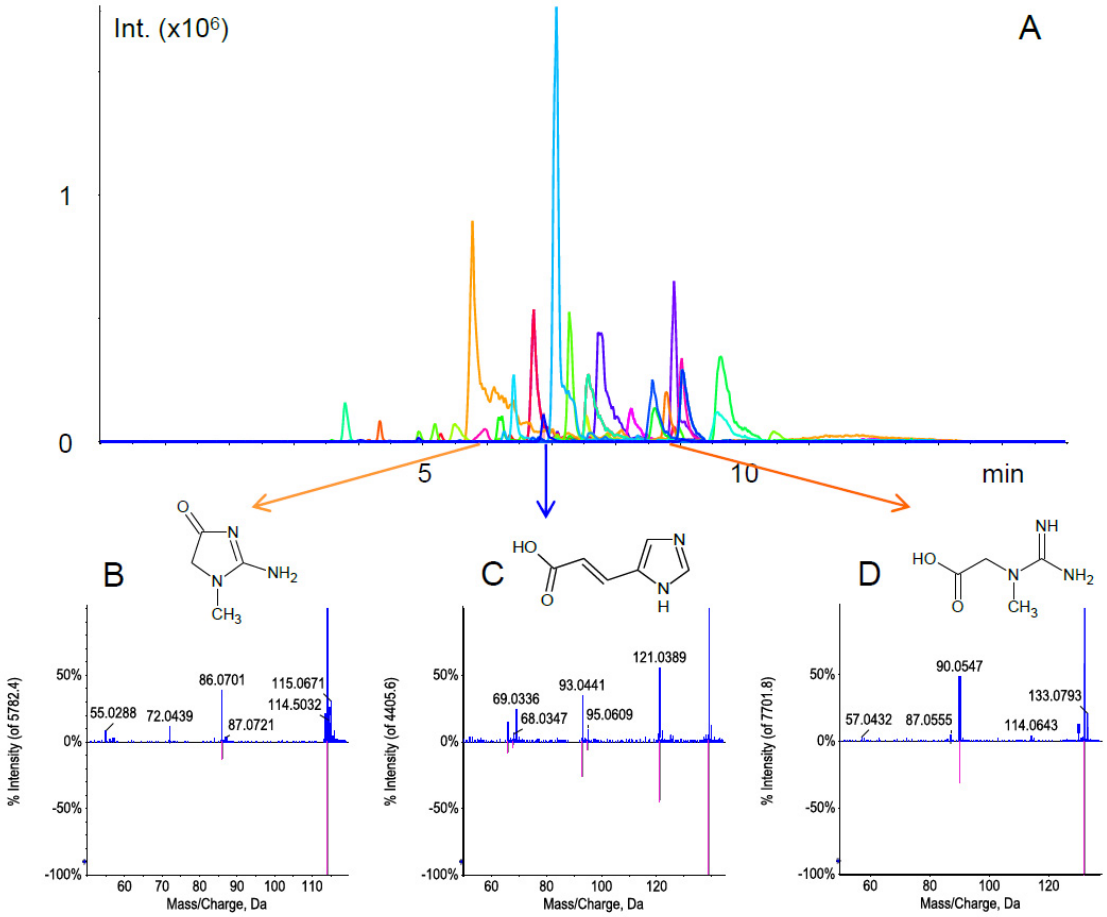

Figure 6. A) Extracted ion current of 74 metabolites identified by SFC-SWATH/MS analysis of a human urine. Experimental spectrum (top) versus composite library spectrum (bottom) for : B) creatinine, C) urocanic acid and D) creatine.

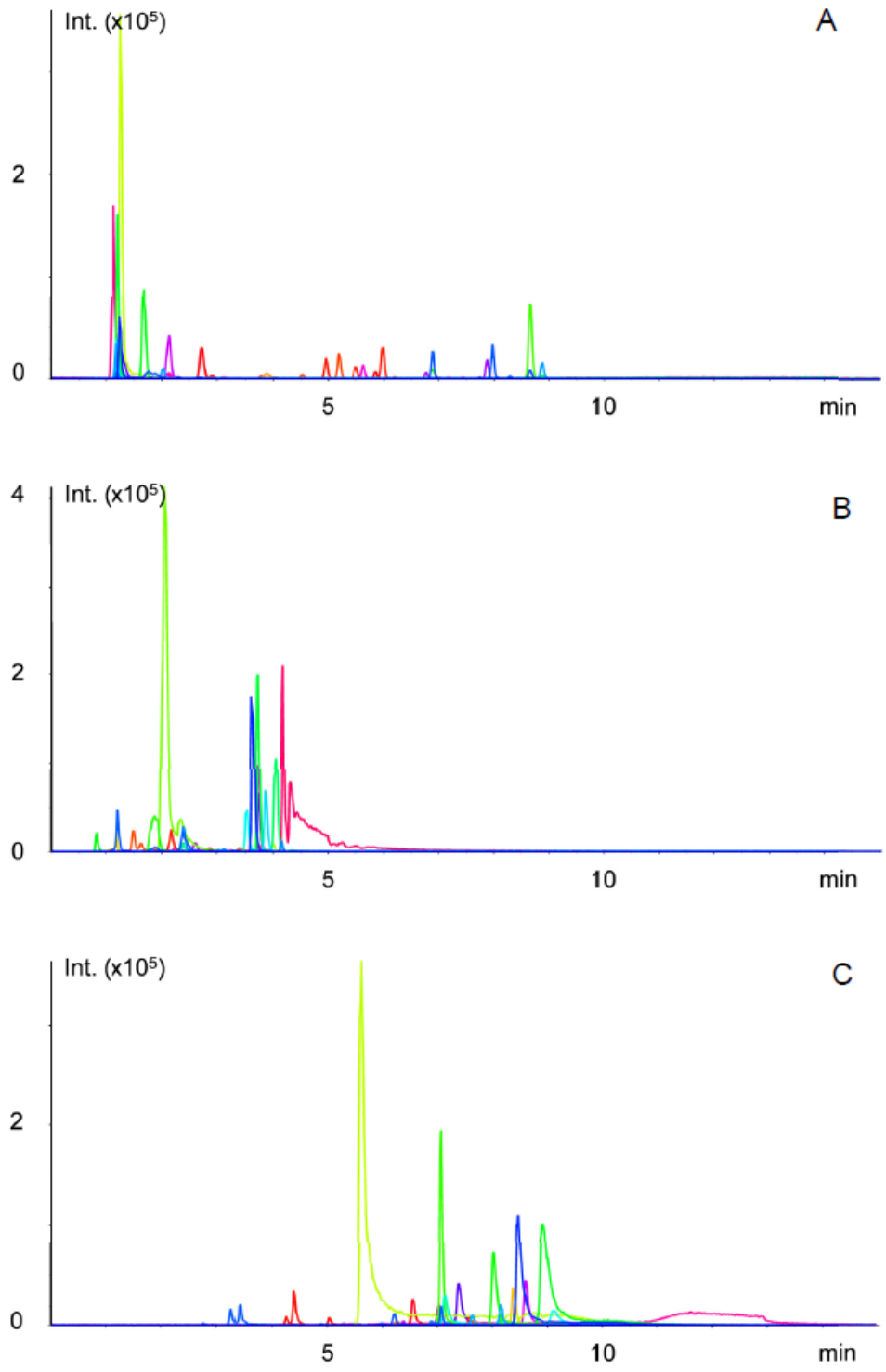

Figure 7. Chromatographic separation of human urine sample in RPLC A), HILIC B) and SFC C) (common identified metabolites, for assignment see ESM, Table S7).