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DESIGN OF STRATEGIES TO STUDY THE METABOLIC PROFILE OF HIGHLY POLAR COMPOUNDS IN PLASMA BY REVERSED-PHASE LIQUID CHROMATOGRAPHY-HIGH RESOLUTION MASS SPECTROMETRY

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Highlights:

- For the first time FMOC is used to label plasma in LC-MS.
- A larger number of FMOC compounds retained in RPLC than underivatized in HILIC.
- Ultrafiltration (UF) generate more plasma features than protein precipitation (PP).
- The UF approach offers better repeatability than the PP method.

Abstract

Amino acids and related compounds are paramount analytes which are involved in numerous metabolic pathways. Most of these compounds are unable to be retained on Liquid Chromatography with Reversed-Phase stationary phases due to their high hydrophilic character. An interesting strategy is to reduce their polarity through their derivatization with a labelling reagent, such as the commercially available 9-fluorenylmethyloxycarbonyl (FMOC) which forms stable complexes with primary and secondary amine moieties rapidly. Although some derivatization reagents have been employed in the study of metabolic profiles, as far as we know, FMOC has never been employed for this purpose. In this work, it is demonstrated that the use of RP-LC~~(C18)-~~MS(TOF) using a C18 column and FMOC as labelling agent enables the determination of a larger number of hydrophilic compounds (proteinogenic amino acids, non-proteinogenic amino acids, and biogenic amines) when compared to the use of a fully-wettable pentafluorophenyl column in fully-aqueous conditions (gradient starting in 0% of organic solvent) and HILIC column, both without using compound derivatization. Different strategies for plasma protein elimination were also carefully evaluated. Results revealed that ultrafiltration (UF) offered a lower variability from sample to

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sample when compared to the protein precipitation (PP) method (from 2 to 12 times lower variability found in UF). Additionally, UF preserved a larger number of possible compounds when compared to the PP approach: 4631 unique molecular features with UF, 666 unique molecular features with PP.

Keywords: 9-fluorenylmethyloxycarbonyl / hydrophilic compounds / liquid chromatography-high resolution mass spectrometry / metabolic profile / plasma / ultrafiltration.

1. Introduction

Amino acids and their metabolites are organic molecules with high importance in many biological pathways, they serve as building blocks for proteins, and they play important roles in neurotransmitter transport and biosynthesis of several other essential biomolecules. As a consequence, they act as biomarkers of different disorders and can even aid in the early detection of a disease, as demonstrated in several metabolomics studies [1-3]. Metabolomics is the latest of the *'omics'* to be implemented after transcriptomics, proteomics, and genomics and it is referred to the study of the metabolome, i.e. the study of low molecular weight compounds which are involved in numerous biochemical processes in a biological system such as biological fluids, tissues, cells, or even in a whole organism [4]. In metabolomics, two main distinctions are made: targeted and untargeted approaches. Targeted approaches aim their efforts on the quantitation of a specific subset of known metabolites, whereas untargeted approaches are focused on the global and unbiased analysis of the small molecules that constitute the metabolome. The latter approach is also known as global metabolite profiling [5]. Metabolic profiles provide useful information related to the state of an

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organism as they are directly related to the cellular activity and are correlated with phenotype in a more direct manner than the rest of the *'omics'* [6].

Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS) are the most used strategies to carry out metabolomics studies, being widely employed for this purpose. NMR is largely used in metabolomics due to its numerous advantages derived from the fact that it requires minimum sample preparation, is rapid, quantitative, nondestructive, offers good reproducibility and high throughput analysis, however, its main drawback is its low sensitivity [7,8]. On the contrary, MS provides much higher sensitivity and it can be easily coupled to a separation technique such as Liquid Chromatography (LC), Gas Chromatography (GC), or Capillary Electrophoresis (CE), enhancing its selectivity and effectiveness. Among these techniques, LC-MS remains the technique of choice in untargeted metabolomics thanks to its robustness, reproducibility, applicability and sensitivity, especially in the reversed-phase (RP) mode [9,10].

Unfortunately, the determination of highly polar compounds, such as amino acids and related compounds, cannot be performed on traditional RP stationary phases, such as C18 columns, due to the fact that they have low or null retention. To solve this issue, different alternative strategies can be carried out, such as the use of polar-embedded and polar-endcapped stationary phases, hydrophilic interaction liquid chromatography (HILIC), the addition of ion-pairing agents to the mobile phase, or even by increasing the hydrophobicity of these compounds by means of derivatization approaches and further analysis by RP liquid chromatography (RPLC). Polar-embedded and polar-endcapped stationary phases have an increased stability in highly aqueous mobile phases due to their hydrogen-bonding ability, offering an alternative selectivity to C18 columns by enabling the retention of polar compounds [11]. HILIC has become

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an attractive alternative to RPLC in the last years thanks to its numerous advantages such as having an increased analyte diffusivity in the organic-rich mobile phase [12], a lower backpressure as a consequence of the low viscosity of the mobile phase [13], and an enhanced MS signal due to the better eluent desolvation [14], among others. Although this approach is perfectly suitable for polar metabolome analysis being often used in combination with RPLC due to their orthogonality [15], HILIC is associated with higher variability in retention times, low peak efficiency, and long re-equilibration times after gradient elution [6,16]. On the other hand, the addition of ion-pairing agents may lead to ion-suppression problems and system contamination [17]. Another option is to increase their hydrophobicity by a derivatization step, so that they can interact with the RPLC stationary phase. Derivatization is an interesting approach not only to improve the chromatographic performance, but also to enhance the sensitivity in MS due to the formation of a compound having higher values of *m/z*. Moreover, derivatization also improves the selectivity due to the possibility of having more characteristic fragments of the compounds under study.

Thus, RPLC methodologies including a derivatization step have been reported in the study of metabolic profiles using different labelling reagents, such as 4-(N,N-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F) [18], 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) [19], phenylisothiocyanate (PITC) [20,21], aminooxy-N-(3-perfluorooctyl-propyl)acetamide [22], the metallic tag bis(2,2'-bipyridine)-4'-methyl-4-carboxybipyridine-ruthenium N-succinimidyl ester-bis(hexafluorophosphate) (Rub2m-*O*-Su) [23], some novel non-commercially available derivatization reagents [24-26], 3,5-dinitrosalicylic acid (DNS) [27-29], and other chemical isotope labelling reagents [30]. Some of these labelling reagents have several drawbacks, for instance, DNS suffers from long reaction times and produces

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photosensitive adducts [19]. The rest of derivatization reagents are either expensive to acquire or are not commercially available. Another reagent which, to the best of our knowledge, has never been employed in a metabolomics study is 9-fluorenylmethyloxycarbonyl chloride (FMOC-Cl). FMOC is well known for reacting almost immediately under mild conditions both with primary and secondary amines, yielding stable compounds [31]. Although FMOC as a labelling molecule has been employed in the determination of amino acids and/or amines in plant roots [32] and forensic fingerprints by LC-MS [33], its application to the analysis of plasma, which is one of the most widely analyzed samples in metabolomics, has been carried out only with UV [34,35] or fluorescence detection [36-38]. Therefore, it could be interesting to evaluate its potential as derivatization reagent in the study of the metabolic profiles of highly hydrophilic metabolites in plasma samples. With this aim, a LC-MS methodology enabling the determination of as many amino acids and amines as possible must be developed, so that it can be extrapolated to the study of hydrophilic compounds in an untargeted approach.

Another key point in metabolomics studies is the sample preparation, in which protein elimination is an often required step due to the fact that proteins severely damage the ionization in mass spectrometry due to ion suppression and they shorten column life. Plasma is known to be a highly protein-rich sample (generally up to 8 g of protein per dL can be found in this matrix [39]). Among the protein elimination procedures used in LC-MS metabolomics studies, the precipitation with organic solvents is the strategy of choice [40]. This protocol also offers the possibility of breaking protein-metabolites bonds so that the metabolite stays free in the sample matrix [41]. However, as it does not eliminate the full protein content from the sample, several problems can occur such as ion suppression, ion source contamination, column

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spoiling, and loss of metabolites if they co-precipitate with the proteins. Other approach to eliminate proteins is the ultrafiltration through cut off membranes (from <3 to <30 kDa). Ultrafiltration advantages comprise obtaining cleaner and more stable samples than protein precipitation although it also has disadvantages such as loss of hydrophobic metabolites and metabolites which are bound to proteins [41].

In this work, the retention behavior of 35 highly polar compounds (amines and amino acids) is studied, first, in polar stationary phases without using a derivatization step, and then, in a C18 column prior FMOC-derivatization. Once the most suitable conditions are found, different sample preparation procedures are evaluated in order to select what is better in terms of extracting the largest number of metabolites from plasma samples with the lowest variability from sample-to-sample.

2. Materials and methods

2.1. Reagents and standard solutions

All reagents were of analytical grade. MS-grade acetonitrile and methanol were obtained from Scharlau Chemie (Barcelona, Spain) while boric acid, formic acid, ammonium carbonate, ammonium hydroxide, sodium hydroxide, pentane, and FMOC were from Sigma (St. Louis, MO, USA). Isoflurane was from Abbott (Madrid, Spain).

Glycine (Gly), γ-aminobutyric acid (GABA), histamine (Him), serotonin (5-HT), taurine (Tau), dopamine (DA), β-alanine (β-Ala), L-alanine (Ala), L-epinephrine (EP), L-histidine (His), L-methionine (Met), L-norepinephrine (NE), L-norleucine (Nle), L-norvaline (Nva), L-arginine (Arg), L-asparagine (Asn), L-aspartic acid (Asp), L-citrulline (Cit), L-cystine (Cys), L-3,4-dihydroxyphenylalanine (DOPA), L-glutamic acid (Glu), L-glutamine (Gln), L-isoleucine (Ile), L-leucine (Leu), L-lysine (Lys), L-ornithine (Orn), L-proline (Pro), L-pyroglutamic acid (Pyr), L-serine (Ser), L-theanine

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(The), L-threonine (Thr), L-tryptophan (Trp), and L-valine (Val) were acquired in Sigma (St. Louis, MO, USA). L-phenylalanine (Phe) and L-tyrosine (Tyr) were from Fluka (Buchs, Switzerland).

A pool containing these 35 compounds dissolved in 300 µL of 200 mM sodium borate (pH 9.0) was derivatized with 300 µL of a solution of 10 mM FMOC in acetonitrile. After 2 min, 600 µL of pentane was employed to eliminate the excess of FMOC, 400 µL from the lower fraction were diluted with 400 µL of water and 200 µL of acetonitrile, and mixed properly, to be then injected into the system, so that the final concentration of each analyte was 27 µM. In the cases where derivatization was not needed the pool of analytes injected had the same final concentration.

2.2. LC-MS conditions

Experiments were carried out using a 1100 series HPLC system (Agilent Technologies, Germany) coupled to a 6530 series quadrupole time-of-flight (QTOF) mass spectrometer (Agilent Technologies, Germany) equipped with a Jet Stream orthogonal electrospray ionization (ESI) source. The HPLC system consisted of a degasser, a quaternary pump, an automatic injector, and a thermostatic column compartment. Agilent Mass Hunter Qualitative Analysis software (B.07.00) was used for MS control, data acquisition, and data analysis. During all analysis, two reference masses were used, *m/z* 121.0509 ($C_5H_4N_4$) and *m/z* 922.0098 ($C_{18}H_{18}O_6N_3P_3F_{24}$) for positive ionization mode, and *m/z* 112.9856 ($C_2O_2F_3(NH_4)$) and *m/z* 1033.9881 ($C_{18}H_{18}O_6N_3P_3F_{24}$) for negative ionization mode, which were continuously infused into the system to allow mass correction.

Analysis was completed at 40 ºC using different columns: two from Ascentis Express (Sigma, St. Louis, USA), with the same dimension (100 x 2.1 mm) and type of

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packed bed (fused-core® particles with 0.5 µm thick porous shell and an overall particle size of 2.7 µm) but having different stationary phases (C18 or HILIC OH5), and a pentafluorophenyl (PFP) Kinetex column from Phenomenex (Torrance, CA, EEUU) of 150 x 3 mm having particles with 0.5 µm thick porous shell and an overall particle size of 2.6 µm. In addition, guard columns (5 x 2.1 mm) of the same material as the analytical column in each case were employed. The system operated with an injected volume of 10 µL and a flow rate of 0.4 mL/min.

The optimal working conditions were obtained with the C18 column, in which the mobile phases consisted of 0.1% formic acid in Milli-Q water (eluent A) and 0.1% formic acid in acetonitrile (eluent B). The linear gradient was set from 5% B to 100% B in 30 min, 100% B for 5 min and returned to starting conditions in 1 min, keeping the re-equilibration at 5% B for 15 min. The ionization source conditions were as follows: capillary voltage of 3000 V with a nozzle voltage of 0 V; nebulizer pressure at 35 psig; sheath gas of jet stream of 6.5 L/min at 275 ºC; and drying gas of 10 L/min at 275 ºC. The fragmentator (cone voltage after capillary) was set at 175 V. The skimmer and octapole voltages were 60 V at 750 V, respectively. MS analysis was performed in positive ESI mode with mass range set at *m/z* 70-1600 (extended dynamic range) in full scan resolution mode at a scan rate of 2 scans per second.

2.3. Animal sampling

Four *Wistar* rats (Barcelona, Spain) were housed at a constant temperature (20 ± 2 °C) on a 12-hour light/dark cycle (lights on at 08:00 hours), with *ad libitum* access to food and water. Animals were maintained and handled according to European Union guidelines for the care of laboratory animals (Directive 2010/63/EU) and the “Principles of laboratory animal care” were followed. All procedures were approved by the

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bioethics committee of the Universidad Nacional de Educación a Distancia (Madrid, Spain). Rats were anesthetized with isoflurane and decapitated, and blood was collected in heparinized tubes, centrifuged for 10 min at 2000 x g (4 ºC) and plasma was collected and stored at −70°C until sample treatment.

2.4. Plasma treatment: protein elimination and derivatization

Protein precipitation (PP) method: acetonitrile was added to plasma (3:1, v/v) and then vortexed for 30 s and left still for an hour in an ice bath. Samples were centrifuged at 10.000xg for 15 min at 4 ºC. 100 µL of 20 mM FMOC in acetonitrile were added to a 100 µL 1:1 (v/v) mixture of plasma to 200 mM sodium borate (pH 9.0). Samples were then vortexed for 2 min and 200 µL of pentane were added to eliminate the excess of labelling reagent [31]. After mixing vigorously, samples were left still for a minute and the lower fraction was taken to be analyzed.

Ultra-filtration (UF) method: a mixture of methanol and 400 mM sodium borate (pH 9.0) (1:1, v/v) was added to plasma (1:1, v/v), then it was vortexed for 30 s and it was left still for 10 min in an ice bath. Samples were centrifuged at 10000xg for 15 min at 4 ºC. The supernatant was vortexed for 30 s to be then ultra-filtered through a 3 kDa cut-off filter (Amicon Ultra Filters, Merck, Darmstadt, Germany) at 14000xg for 25 min at 4 ºC. 100 µL of the filtrate were mixed with 100 µL of 20 mM FMOC in acetonitrile. As in the protein precipitation method, samples were then vortexed for 2 min and 200 µL of pentane were added to eliminate the excess of labelling reagent [31]. After mixing vigorously, samples were left still for a minute and the lower fraction was taken to be analyzed.

2.5. Data processing and statistical analysis

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Molecular Feature Extraction (MFE) tool from Mass Hunter Qualitative Analysis (B.07.00) was employed to obtain time and mass abundances coming from chromatographic peaks. MFE algorithm was used to group ions related by charge-state envelope, isotopic distribution and the presence of adducts (H^+, Na^+, and K^+ in positive ionization mode and $HCOO^-$ in negative mode), dimers and ions that belong to the same compound (e.g. FMOC species). Other parameters include "small molecules (chromatographic)" as extraction algorithm, ions with more than 500 counts were considered, peak spacing tolerance=0.01 *m/z*, plus 10.0 ppm; isotope model=common organic molecular; limited assigned charge =2.

Once molecular features were extracted; alignment and filtering were carried out by means of the Agilent Mass Profiler Professional tool (B.02.00). Retention time data range was set from 2 min; mass tolerance 15 ppm with a mass window of 0.02 Da and a retention time window of 0.2. Multivariate statistical analysis was carried out using SIMCA 13.0 (Umetrics, Umeå, Sweden).

3. Results and discussion

3.1. Study of the interaction of the studied hydrophilic analytes with different stationary phases

It is well known that highly polar compounds cannot be retained in RPLC columns and, as a result, they elute with the column dead volume making it difficult to carry out the proper determination in a complex matrix. It is a fact that in most of samples with high importance, such as biological samples (e.g. urine or plasma [43]) or even samples from other sources (e.g. foodstuffs or beverages [44]), the amount of polar compounds is elevated. Thus, the development of analytical methodologies enabling the determination of highly polar compounds in this kind of matrices is necessary being LC

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with high resolution MS detection a powerful tool to face this task due to its robustness, high sensitivity and excellent selectivity.

In this work, a group of 35 highly polar compounds that comprise proteinogenic and non-proteinogenic amino acids, and several biogenic amines (see *section 2.1*) was selected. In order to determine these compounds by LC-MS, their interactions with different stationary phases under different chromatographic conditions were investigated using a pool containing standard solutions of the 35 studied compounds. The purpose of this optimization protocol was to obtain the optimal conditions for the retention and separation of these compounds but under general gradient conditions so that it can be further applied to carry out untargeted metabolomics studies. The interaction of the underivatized analytes was evaluated in columns with less hydrophobicity than the standard C18, such as pentafluorophenyl (PFP) and HILIC, and the results obtained were compared with those corresponding to the determination of the FMOC-labeled analytes in a C18 column.

The first column evaluated was a PFP column, a fully-wettable column that offers different interactions mechanisms such as hydrophobic, π-π, electrostatic, and hydrogen bond interactions, and that is suitable both for low-polar and mid-polar compounds. Regarding this column, fully-aqueous conditions can be employed, so first a gradient from 0 to 100 % of B in 30 min being eluent A water and eluent B acetonitrile, both with 0.1 % formic acid, was tested. This set of conditions enabled the retention of only 12 of the 35 compounds studied, 17 compounds had no retention (they eluted with the dead column volume), and 6 compounds were not detected. In order to improve retention in the PFP column, methanol was used as organic modifier instead of acetonitrile. The new mobile phase based on methanol offered better retention and 16 of the 35 compounds studied were retained, 13 compounds had no retention, and 6

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compounds were not detected. Once methanol was selected as organic solvent in phase B with the PFP column, 10 mM ammonium acetate was used instead of formic acid, i.e. more basic conditions, and this new mobile phase offered a better retention as 20 of the 35 compounds studied were retained, whereas that 14 compounds had no retention, and only 1 compound was not detected.

The second column evaluated was a HILIC column using a method reported by Qi et al. for determination of polar compounds [45], thus a gradient elution from 100 to 55 % of B, being the eluent A 10 mM ammonium formate and 0.2 % formic acid in water and the eluent B 95% acetonitrile and 5% of 2 mM ammonium formate and 0.2 % formic acid in water ~~[45]~~. It is remarkable to point out that when comparing the best conditions of PFP and the conditions described for HILIC, HILIC offered a better analytical performance as it enabled to obtain a larger number of compounds retained in this stationary phase (29 of the 35 studied compounds), although the signal intensity was higher in the case of the PFP column.

Due to the fact that none of the tested conditions enabled the retention of all the compounds of interest another strategy had to be assayed. As stated in the introduction, by labelling the studied analytes their hydrophobicity can be enhanced and therefore their interaction with a RPLC stationary phase, a traditional C18 column, can be stronger resulting in their retention onto this kind of stationary phases. Here we studied the potential of FMOC to enhance the retention of the studied analytes due to its numerous advantages as forming stable compounds rapidly. Chromatographic conditions consisted of a gradient from 5 to 100 % of B in 30 min being eluent A water and eluent B acetonitrile, both with 0.1 % of formic acid. These new conditions offered the best results since 33 of the 35 compounds studied were retained (only The and Pyr were not detected), this being the largest number of compounds found so far. Also, note

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that under these conditions isomeric molecules could be perfectly separated (Leu/Ile/Nle, Val/Nval, and Ala/β-Ala), unlike under any of the conditions previously tested. As expected, when comparing the peak shapes obtained with the other columns, the efficiency of the strategy based on the C18 column with FMOC-derivatization was far better than the one obtained with the underivatized analytes in the HILIC and PFP conditions. As expected, the retention of the studied compounds was substantially greater in the C18 column (all compounds eluted between 9 and 19 min) when compared to the PFP (compounds eluted between 2 and 11 min) and the HILIC (compounds eluted between 5 and 14 min). In addition, the fact that the studied compounds in C18 eluted at higher % of the eluent B also implied a sensitivity improvement as a higher content of organic solvent in the mobile phase is associated with higher MS ionization efficiency.

Regarding the MS conditions employing the C18 column, the number of compounds in positive (mobile phase with 0.1 % of formic acid) and negative (mobile phase with 10 mM ammonium acetate) ionization modes was not so different, but the sensitivity obtained in the positive mode was much higher for all analytes except for Ser, Tau and Asp whose sensitivity was slightly higher in the negative mode. According to the obtained results, we concluded that the best conditions for the C18 column were the ones in the positive ionization mode. Under these conditions, it is important to note that the chemical FMOC-labelling provides a signature that is easily distinct by MS as the studied compounds were detected as ions derived from the complex with FMOC ($[M+FMOC+H]^+$, $[M+FMOC+Na]^+$ and $[M+FMOC+K]^+$) whereas the $[M+H]^+$ specie was observed for all the studied compounds except for Arg, Cys, Orn, His, and Him. This is of special importance due to the fact that in an untargeted metabolomic strategy the molecular feature extraction algorithm needs to detect both, the $[M+H]^+$ and the

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[M+FMOC]-derived species to identify them as the same compound. Thus, in order to also obtain the $[M+H]^+$ the temperature of the ion source and cone voltage after sampling capillary and before the ion optics (called fragmentator) needed to be optimized. Different fragmentator voltages (50, 100, 175, and 225 V) and sheath gas and nebulizer temperatures (275, 300, and 325 ºC) were investigated where 175 V and 275 ºC proved to be the optimal conditions as it was the only combination in which all of the 35 compounds could be finally detected, in addition to the detection of the $[M+H]^+$ specie of every single analyte (see **Fig. 1A**). Note that this optimization was also carried out for non-derivatized analytes in the HILIC column to see if we could increase the number of detected analytes but this was not the case. The best conditions of HILIC, in terms of the number of compounds determined and sensitivity were the initial conditions (100 V of fragmentator and 300 ºC of sheath gas and nebulizer temperatures) (see **Fig. 1B**).

As shown in **Fig. 1A**, two different retention time windows are well differentiated, one from 9 to 19 min in which all 35 compounds derivatized with a molecule of FMOC eluted, and the other window, from 20.5 to 22 min for those analytes which reacted with two molecules of FMOC (Cys, Orn, His, and Lys). This means that in a time window of only 10 mins, 35 compounds could be determined (for those derivatized with a molecule of FMOC), so in case only these compounds were to be followed, for example, in a targeted strategy, gradient can be modified to speed up the chromatographic separation. However, as the aim of this work was to propose a method to separate highly polar compounds for a future untargeted metabolomics approach, general linear gradient conditions were employed.

3.2. Treatment of plasma samples

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Due to the fact that plasma reflects global changes in the metabolism, rat plasma was chosen as test sample to show the versatility of the developed LC(C18)-MS methodology employing FMOC as derivatization reagent.

First, the concentration of FMOC necessary to label all the compounds existing in the sample was studied. 50 µL of plasma were mixed with 50 µL of 400 mM borate (pH 9.0) and were derivatized with 100 µL of FMOC in acetonitrile at different concentrations (from 1 to 40 mM). The studied compounds had nearly the same behavior: peak area increased until 5-10 mM of FMOC and then it was kept practically constant until 40 mM. Therefore, we selected 20 mM to ensure that all amino-containing analytes were completely derivatized. It is important to highlight that FMOC derivatization was completed in just 2 min at room temperature, this being the main advantage of FMOC when compared to other derivatization reagents that have been used in metabolomics-related studies. DBD-F needed reaction times of 30 min at 30 ºC [28], AQC 15 min at 55 ºC [19], aminooxy-N-(3-perfluorooctyl)acetamide overnight at 70 ºC [22], Rub2m-*O*-Su 1.5 h at 55 ºC [23], and DNS 1 h at 60 ºC [27,28]. Exposing samples to high temperatures could, additionally, lead to thermal degradation of the metabolites of interest, so working at room temperature should offer a better sample stability.

Second, the procedure to eliminate proteins from plasma was investigated. As stated in the introduction, PP is the strategy most widely employed even though UF offers promising results [41]. As both strategies offer advantages the use of UF with a previous step of addition of organic solvent enough to break metabolite-protein bonds was investigated and compared with the widely applied method based on PP with an organic solvent. Thus, four rat plasma samples were pooled and, on the one hand, five

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aliquots were individually treated by the PP method and, on the other hand, another five aliquots of plasma were treated by the UF method, both according to *section 2.4*.

Fig. 2 shows the total ion chromatograms (TICs) of a representative analysis from each treatment where it is noticeable that the TIC signal of the UF sample is higher than that of the PP procedure, this is mainly caused by the fact that plasma treated by the PP method had to be diluted four times with organic solvent, this being the main drawback of the PP strategy. Moreover, **Fig. 3a** shows the extracted chromatogram of the 35 investigated hydrophilic compounds in a rat plasma sample treated with the UF strategy in which 30 out of 35 compounds studied could be detected (all except DA, DOPA, EP, Nle and The). Note that the PP strategy also offered a lower number of the followed compounds was obtained, as Him, Cys, Tau, GABA, NE, and Nval could not be detected (see **Fig. 3b**), compounds which could be determined in samples treated with the proposed UF strategy.

On the other hand, molecular features from both groups (UF and PP), extracted according to *section 2.5*, were filtered by retaining those components having a signal larger than 10000 counts, which were present in all the samples of each group, and whose RSD % was not higher than 35 %. **Fig. 4** displays the Venn diagram with the features that met these filtering conditions and that are unique and common for the two sample treatments, where it can be seen that the UF approach enabled to obtain up to 7-times more unique features than the PP treatment (4631 *vs* 666). Additionally, when plotting a principal component analysis (PCA) of the common features found in the samples studied (745 features), it can be observed the clear separation among these two groups in the first principal component but, above all, it is noteworthy the fact that the UF samples clustered tighter than samples of PP that remained more dispersed. This clearly demonstrated that the features obtained by the UF approach had lower variability

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from sample to sample when compared to the PP method. Additionally, when taking into account the RSD of peak areas of the group of amino acids and amines that were followed in this work and that were present in both strategies, again, it can be seen how the UF method offered much lower variability when compared to PP (from 2 to 12 times lower variability found in UF for these compounds, except for β-Ala whose RSD was slightly higher in UF) (see **Table 1**). Due to the fact that the UF allowed to obtain a larger number of molecular features with lower variability, attributes of high importance in any metabolomics approach, it was chosen for further studies.

3.3. Precision and sample stability assessment

An important issue to take into account is to evaluate the precision and the sample stability of the developed strategy, as when metabolomics studies need to be carried out, precise methods must be used to assure that the differences are due to real biological variabilities and not to instrumental or methodological fluctuations. Thus, instrumental and method repeatability, intermediate precision, and sample stability were determined for the 30 compounds found in the ultra-filtered plasma.

First, instrumental repeatability was assayed as the triplicate injection of a derivatized plasma sample. As shown in **Table 2**, RSD values for all peak areas were generally below 5 % and below 0.2 % for the retention times (RT). Regarding method repeatability, evaluated from the injection in the same day of three individual plasma samples individually derivatized, RSD values for peak areas were below 10.8 % whereas that in the case of RT, once again, they were below 0.2 %. The intermediate precision in a three days assessment was evaluated as the duplicate injection of a sample derivatized each of the three days. RSD values of peak areas were in all cases below 15

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% except for Asp which was slightly higher, and again, RSD values for RT were below 0.2 %.

Another important aspect is to study how stable plasma samples are at room temperature once they have been derivatized with FMOC. To address this issue, a single plasma sample was derivatized and was injected six times in a time span of 16 h. Except for few exceptions, RSD values of peak areas were below 10 %, and in all cases RSD values for RT were below 0.2 % (see **Table 2**), thus, samples are considered stable at least for 16 h at room temperature. This highlights the suitability of the proposed methodology to be applied in metabolomics studies.

4. Concluding remarks

In this work, the use of FMOC as labelling agent is shown to offer better performance in the determination of a group of 35 polar compounds (amino acids and amines) in a C18 column, when compared to other polar stationary phases such as PFP or HILIC, not only in number of retained compounds but also in terms of sensitivity and peak efficiency, under the assayed conditions.

The developed LC(C18)-MS methodology was applied to the analysis of plasma samples, due to the high interest in the analysis of this matrix, especially in the metabolomics field. It was found that the ultrafiltration strategy enabled to obtain a larger number of compounds with a lower variability from sample to sample when compared to the protein precipitation method. The three different precision studies (instrumental and method repeatability, and intermediate precision) and the stability study performed on the plasma samples gave adequate results. Therefore, it has been demonstrated that the proposed methodology is superior to the other assayed conditions

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regarding plasma treatment and chromatographic conditions in the detection of polar compounds.

Conflicts of interest

Authors declare that they have no conflict of interest

Acknowledgments

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Figure captions.

Fig. 1. Extracted Compound Chromatograms obtained under the optimal conditions for (A) FMOC-derivatized analytes in C18 and (B) non-derivatized analytes in HILIC. Chromatographic conditions are detailed in the text. xLeu and xVal are used when identification of the isomers of these compounds was not carried out.

Fig. 2. Total Ion Chromatograms (TIC) of the same rat plasma sample deproteinized by protein precipitation (PP) and ultrafiltration (UF).

Fig. 3. Extracted Compound Chromatogram of the investigated hydrophilic compounds in a plasma sample treated with (a) ultrafiltration strategy and (b) protein precipitation strategy, both analyzed by means of the LC(C18)-MS methodology.

Fig. 4. Venn diagram displaying the unique and common molecular features found in the protein precipitation and ultrafiltration strategies, and PCA score plot having the common features for the two different treatments as variables.

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Fig. 1.

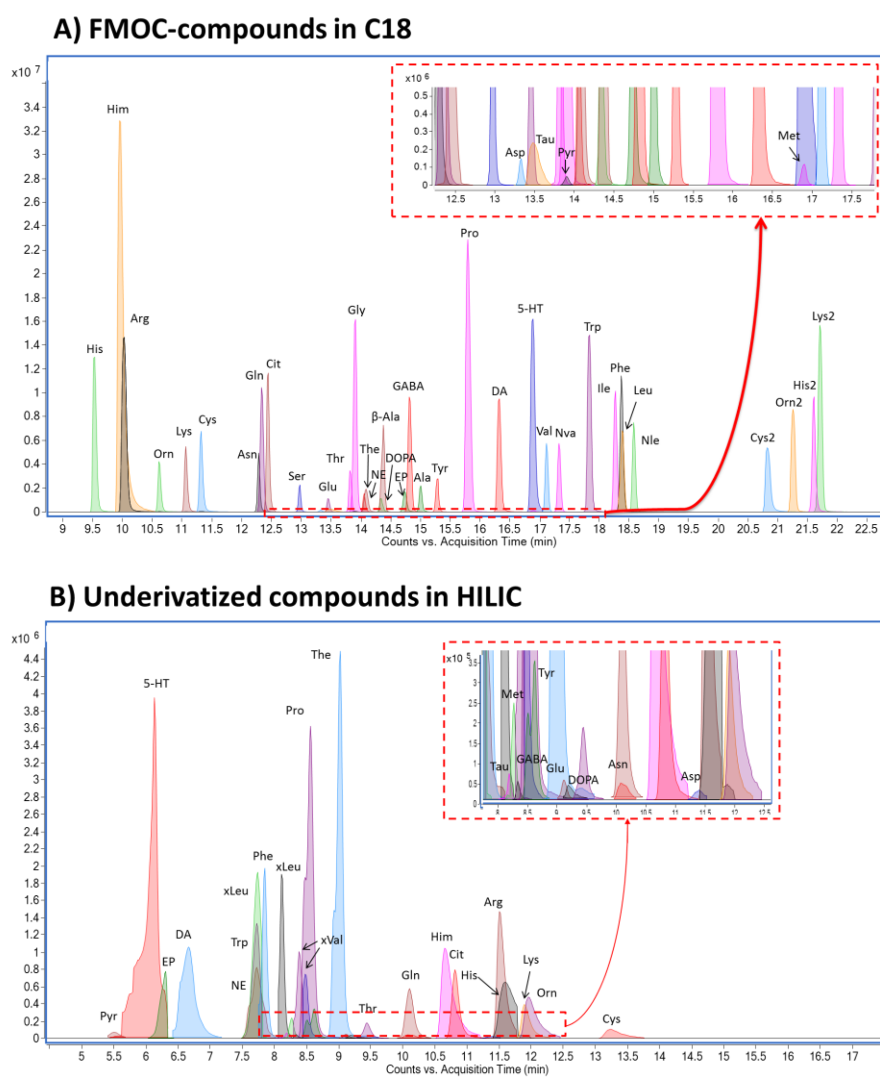

Fig. 2.

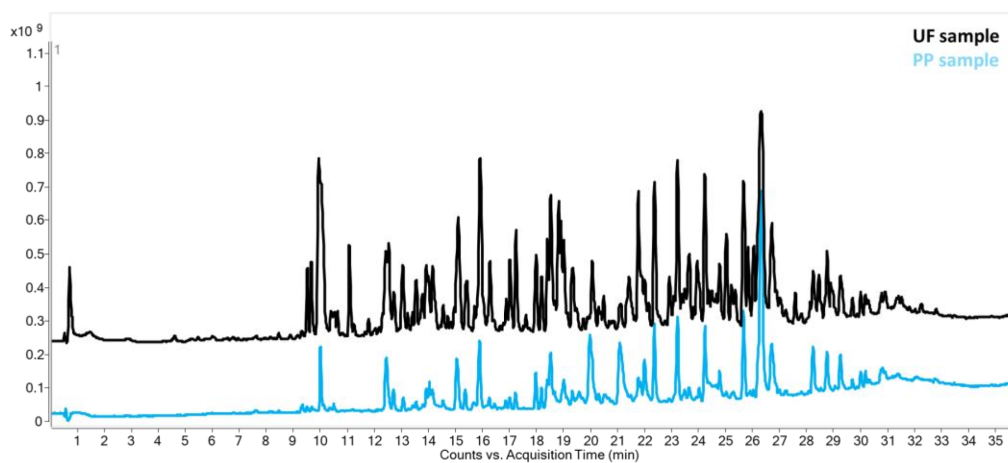

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Fig. 3.

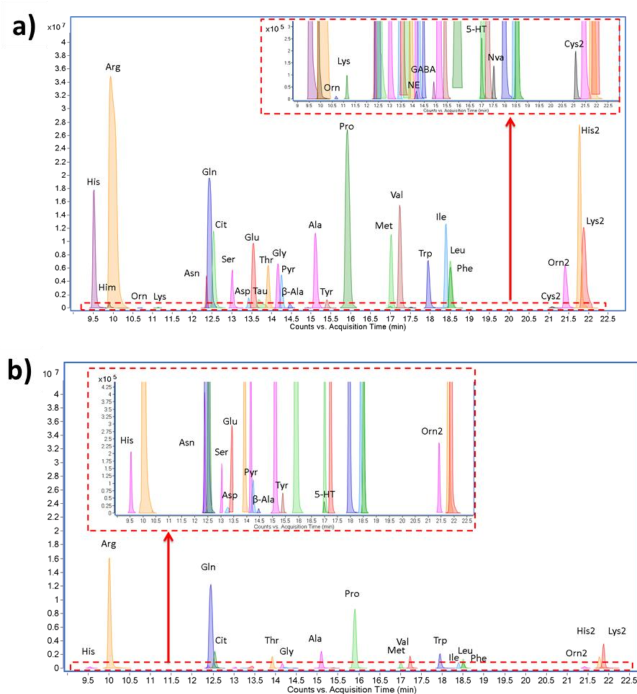
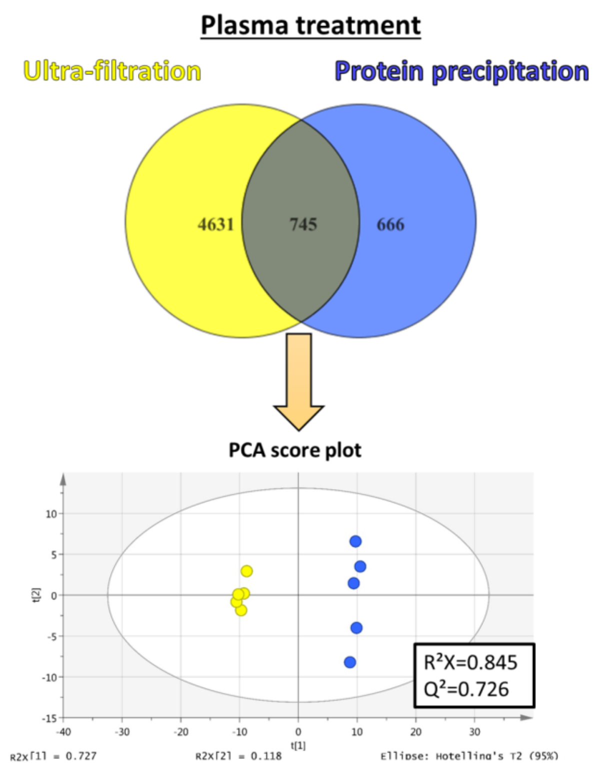


Fig. 4.



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Tables

Table 1. RSD (%) values obtained for peak areas for the compounds determined in plasma by the PP and UF methodologies.

Analytes	RSD (%) of peak areas (n=5)	
	PP	UF
Alanine	8.4	3.6
β-alanine	9.1	10.0
Arginine	16.6	3.6
Asparagine	13.4	3.9
Aspartic acid	18.7	8.0
Citrulline	17.2	3.1
Glutamic acid	24.4	2.3
Glutamine	11.1	1.3
Glycine	14.1	8.3
Histidine	43.7	21.3
Lysine	24.8	2.0
Methionine	25.9	3.8
Leucine	23.4	11.8
Isoleucine	20.9	3.8
Ornithine	32.6	8.8
Phenylalanine	22.8	8.3
Proline	13.5	1.6
Pyroglutamic acid	47.7	6.1
Serine	20.5	4.1
Serotonin	41.4	9.6
Threonine	9.1	5.5
Tryptophan	14.5	6.2
Tyrosine	20.6	5.5
Valine	14.2	3.7

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Table 2. RSD (%) values obtained for peak areas and retention times (RT) for the compounds determined in plasma in the instrumental and method repeatability, intermediate precision and the 16 h stability studies.

Analytes	RSD (%)							
	Instrumental repeatability (n=3)		Method repeatability (n=3)		Intermediate precision (n=6)		16 h stability (n=6)	
	Peak area	RT	Peak area	RT	Peak area	RT	Peak area	RT
Alanine	0.9	0.03	0.7	0.1	5.1	0.1	3.1	0.2
β-alanine	2.8	0.1	5.2	0.1	9.6	0.03	11.6	0.2
Arginine	1.8	0.04	2.7	0.1	8.6	0.2	6.7	0.2
Asparagine	1.0	0.04	1.1	0.1	8.8	0.1	6.7	0.1
Aspartic acid	2.0	0.1	4.3	0.1	17.7	0.1	9.3	0.2
Citrulline	1.5	0.1	2.3	0.2	7.7	0.1	4.6	0.1
Cystine	1.8	0.02	1.2	0.1	7.3	0.1	5.5	0.1
GABA	4.4	0.1	10.3	0.1	13.2	0.1	14.1	0.2
Glutamic acid	0.8	0.1	1.5	0.1	11.1	0.1	4.2	0.2
Glutamine	0.6	0.2	0.7	0.1	2.8	0.03	2.7	0.1
Glycine	1.6	0.1	1.3	0.1	10.4	0.1	5.4	0.2
Histamine	8.9	0.1	10.6	0.1	12.3	0.1	10.9	0.1
Histidine	4.3	0.1	7.0	0.05	12.0	0.1	7.9	0.2
Lysine	1.0	0.02	2.8	0.1	3.7	0.1	2.9	0.04
Methionine	0.9	0.02	3.7	0.1	7.9	0.05	5.6	0.1
Norepinephrine	2.0	0.1	8.0	0.1	11.2	0.1	6.3	0.2
Norvaline	5.2	0.04	5.3	0.1	12.5	0.1	8.8	0.1
Leucine	4.3	0.02	7.5	0.1	8.5	0.1	5.2	0.1
Isoleucine	3.0	0.04	4.0	0.04	9.3	0.1	4.2	0.1
Ornithine	4.7	0.1	6.9	0.1	12.7	0.1	11.6	0.1
Phenylalanine	2.0	0.1	5.1	0.1	7.4	0.1	4.5	0.1
Proline	0.3	0.02	0.4	0.1	8.7	0.04	3.9	0.1
Pyroglutamic acid	2.0	0.1	3.1	0.1	12.4	0.1	8.2	0.2
Serine	1.2	0.1	1.1	0.1	4.5	0.1	2.0	0.2
Serotonin	5.2	0.04	7.9	0.1	12.1	0.1	7.2	0.1
Taurine	0.9	0.1	2.0	0.1	15.2	0.1	8.4	0.2
Threonine	1.7	0.03	8.0	0.1	11.6	0.1	7.8	0.2
Tryptophan	1.3	0.02	6.3	0.1	6.8	0.03	5.0	0.1
Tyrosine	3.8	0.03	10.8	0.1	9.7	0.1	13.5	0.2
Valine	0.1	0.04	1.7	0.1	6.9	0.1	4.9	0.1