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Evaluation of human plasma sample preparation protocols for untargeted metabolic profiles analyzed by UHPLC-ESI-TOF-MS

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Abstract

Eight human plasma preparation protocols were evaluated for their suitability for metabolomic studies by ultra-high performance liquid chromatography coupled to electrospray ionization time-of-flight mass spectrometer (UHPLC-ESI-TOF-MS): organic solvent protein precipitation (PPT) with either methanol or acetonitrile in 2:1 and 3:1 (v:v) organic solvent:plasma ratios, solid phase extraction (SPE) using C18 or Hybrid SPE cartridges, a combination of PPT and SPE C18 cartridges and microextraction by packed sorbent. A design of the study in which the order of injection of the samples was not randomized is presented. The analyses were carried out in a BEH C18 column (1.7 μm, 2.1 x 100 mm) using a linear gradient from 100 % water to 100% methanol, both with 0.1 % formic acid, in 21 min. The most reproducible protocol considering both univariate and multivariate results was PPT with acetonitrile in a 2:1 (v:v) organic solvent:plasma ratio, offering a mean coefficient of variation of the area of all the detected features of 0.15 and one of the lowest Euclidean distances. On the other hand, the highest number of extracted features was achieved using methanol in a 2:1 (v:v) ratio as PPT solvent, closely followed by the same protocol with acetonitrile in a 2:1 (v:v) proportion, which only offers 1.2 % less repeatable features. In terms of concentration of remaining protein, protocols based on PPT with ACN provided cleaner extracts than protocols based on PPT with methanol. Finally, pairwise comparison showed that the use of PPT and SPE based protocols, offers a different coverage of the metabolome.

Keywords: Metabolomics, plasma, sample treatment, reproducibility, Liquid chromatography-mass spectrometry, Hybrid SPE.

1. Introduction

Metabolomics consists of the untargeted analysis of low molecular weight metabolites (< 1 kDa) in biological samples [1-4]. The principal aim of this omic science is to provide an insight into the metabolic status of complex living systems. Comparison of the metabolic profiles from different phenotypes can be used to identify specific metabolic changes leading to the understanding of physiology, toxicology and disease progression [5, 6].

The more widespread analytical platforms in metabolomics are nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) coupled to liquid chromatography (LC) or gas chromatography (GC) [7]. Although NMR is more robust, repeatable and needs a simpler sample treatment, which is ideal for high throughput analysis, this technique has an important drawback that is overcome by MS, its low sensitivity. Historically most metabolomic studies were carried out using GC coupled to mass spectrometry, but its limitations related to the molecular weight and type of metabolites that can be analyzed and the extensive preparation required led to the emergence of liquid chromatography coupled to mass spectrometry (LC-MS) as a metabolite profiling tool. When MS is coupled to ultra-high performance liquid chromatography (UHPLC), it is able to analyze simultaneously thousands of metabolites requiring a small sample volume for analysis. Nonetheless, MS does not provide as much structural information as NMR, being more difficult to accomplish the identification of biomarkers. As the aforementioned techniques have different strengths and drawbacks, the ideal case would be to analyze the same sample set by a combination of them [7, 8].

In all fields of analytical chemistry it is vital to use a repeatable sample preparation protocol in order to minimize the differences between samples due to the analytical process. This is especially important when human samples are analyzed because the influence of diet, environmental effects as well as genetic-related factors causes high interindividual variability itself. In a metabolomic study two different approaches can be used for the comparison of different sample treatment protocols, univariate and multivariate analysis. Using a traditional statistic approach the median and mean coefficient of variation (CV) of the intensities of all detected features (pairs mass-to-charge ratio (m/z)-retention time (RT)) can be calculated among the

replicates of each of the different protocols. It is also possible to know the percentage of features that has a CV value lower than a certain value among the replicates, for example 0.3, which is considered the maximum acceptable value for the total error for targeted LC-MS analysis [9], and the percentage of features present in all (% N all) or in a certain number of replicates. The comparison of these values between protocols gives an overview of the differences in repeatability. However, it is important to bear in mind that metabolomic data sets are complex matrices composed by thousands of features that should be also analyzed using a multivariate approach to combine multiple features and enhance the statistical comparison of methods. Principal component analysis (PCA) is the most suitable analysis for this purpose. One way of analyzing the repeatability in PCA is calculating the intrareplicate distance within the model, Euclidean distance (ED), value that gives a comparison of relative repeatability among the replicates.

As in metabolomics the general aim is to study as many metabolites as possible, the best sample preparation would be the one in which the sample is modified as little as possible (for example a simple dilution is the usual protocol for urine metabolomic studies [10]). Previous works have reported the study of sample treatment of matrices such as liver [11, 12], plasma [13-18], cells or microbes [19-24], feces [25] by different analytical techniques. In the case of plasma samples direct injection is not possible without the rapid degradation of the columns and loss of sensitivity caused by the gradual build-up of non-volatile compounds in the cone of the ionization source. For these reasons and due to the important ion suppression caused by endogenous compounds in LC-MS, it is crucial to apply a clean-up step before the analysis. This is usually achieved by protein precipitation (PPT) using organic solvents, being the most employed methanol (MeOH) or acetonitrile (ACN). An alternative approach that does not involve necessarily PPT and thus allows only the study of non strongly protein-bound metabolites is solid phase extraction (SPE). A combination of organic solvent precipitation of proteins and SPE (SPE + PPT) can be used with the aim of releasing metabolites bound to proteins and prolonging column life. These SPE approaches remove substances that are prone to being strongly retained on the column if the same stationary phase is used. Taking into account that one of the major challenges in metabolomics is the analysis of a great number of plasma samples, the removal of these substances could be very helpful in order to keep the ionization source clean and lengthen the column life. However, to our knowledge, SPE has only been applied in one

metabolomic study of plasma samples analyzed by LC-MS [17]. Finally another clean-up option is offered by phospholipids removal cartridges such as Hybrid SPE. The packed-bed filter/frit assembly of these cartridges acts as a depth filter for the concurrent physical removal of precipitated proteins and chemical removal of phospholipids while small molecules pass through unretained, giving as a result a cleaner sample in which ion suppression caused by phospholipids is eliminated [26]. Depending on the specific aim of each study different sample preparation protocols should be considered.

In this study eight human plasma preparation protocols were evaluated for their suitability for UHPLC–electrospray ionization (ESI) time-of-flight (TOF) MS based untargeted metabolomics: four of them based on organic solvent protein precipitation with either ACN or MeOH in 2:1 and 3:1 (v:v) organic solvent:plasma proportions, two approaches in which SPE C18 cartridges were used, with and without previous protein precipitation step, Hybrid SPE cartridges and microextraction by packed sorbent (MEPS). To our knowledge this is the first time that the effectiveness and repeatability of Hybrid SPE and MEPS are evaluated in human plasma metabolomics. Other plasma sample preparation protocols, as lowering pH with acid or protein denaturation using heat, were discarded from the beginning because according to Want et al [15] the number of extracted features obtained with these protocols is considerably low. The aim of this work was, therefore, to study these different human plasma preparation protocols by means of univariate and multivariate analyses in order to select a reliable and repeatable sample preparation protocol for the untargeted metabolic analysis of human plasma that offers the highest number of extracted features. Other parameters as number of extracted features, reproducibility of a series of selected features and the amount of protein remaining in each reconstituted plasma extract were also studied.

2. Materials and Methods

2.1 Reagents and materials

Solvents for LC-MS and sample preparation (MeOH and ACN) were OPTIMA® LC-MS grade and were obtained from Fisher Scientific (Los Angeles, CA, USA). Formic acid (FA) and sodium formate, both LC-MS grade, leucine enkephalin (HPLC grade), bovine serum albumin solution from (BSA) and Bradford reagent were from Sigma-Aldrich (St. Louis, MO, USA). Purified water from a Millipore (Milford, MA,

USA) Milli-Q Element A10 water system was used along the study. For UHPLC-ESI-TOF-MS analysis, 96-well plates were from Waters (Milford, MA, USA); well plate cap mats were purchased from VWR International (Leicestershire, UK); Hybrid SPE precipitation cartridges were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Strata-X SPE polymeric cartridges were from Phenomenex (Torrance, CA, USA). For carrying out MEPS extraction an eVol® XR hand-held automated analytical syringe coupled to a 500 µL MEPS syringe that contains a MEPS C18 cartridge, all purchased from SGE Analytical Science (Victoria, Australia), was used.

2.2 Plasma samples

Blood samples from ten healthy volunteers were collected into EDTA tubes. Written informed consent was obtained from all the volunteers. Plasma samples were prepared by centrifugation in an Eppendorf 5804 R centrifuge (Hamburg, Germany) at 1200 g at 4 ºC for 10 min. Pooled samples were prepared mixing equal amounts of the 10 plasma samples into one vessel. This plasma pool was divided in 10 mL fractions that were used to evaluate the different sample preparation protocols. All the samples were stored at -20 ºC until the analysis.

2.3 Plasma sample preparation

Frozen plasma was thawed on ice. Eight sample preparation protocols were compared (Fig 1), each using 6 preparation replicates and 3 instrumental replicates. All the samples extracts at the end of the treatment contained the same proportion plasma:reconstitution solvent (MeOH:H_2O 50:50 v:v).

2.3.1 Organic solvent PPT

This type of sample preparation consisted of a protein precipitation with either cold MeOH or ACN. The starting volume of plasma was 250 µL. Two different ratios of organic solvent:plasma were tested, 2:1 and 3:1 (v:v). After brief vortexing, plasma samples were centrifuged at 13000 g for 5 min. 600 µL and 800 µL of the supernatant were taken for 2:1 and 3:1 (v:v) ratios, respectively, and they were dried under a stream of N_2 at 40 ºC in a Zymark Turbovap evaporator (Barcelona, Spain). Finally, the dried extracts were reconstituted in 200 µL of MeOH:H_2O 50:50 (v:v).

132 2.3.2 Solid phase extraction procedure

133 Two SPE approaches were studied, one included a protein precipitation step prior to the extraction and the
134 other a simple dilution of the plasma sample with H_2O. The protein precipitation was carried out with
135 MeOH in a ratio 3:1 (v:v) MeOH:plasma, as it was detailed in the previous section, but instead of
136 evaporating the supernatant to dryness, when the volume was less than 200 µL, 800 µL of H_2O were added.
137 In the case of the dilution with H_2O, 800 µL of H_2O were added to 200 µL of plasma. SPE cartridges were
138 previously activated with 1 mL of MeOH and then further conditioned with 1 mL of H_2O. Diluted samples
139 were loaded into the cartridge and cleaned with 1 mL of H_2O. Subsequently, 1 mL of MeOH was used as
140 elution solvent. Finally, the eluate was dried under N_2 stream at 40 ºC and reconstituted in 200 µL of
141 MeOH:H_2O 50:50 (v:v).

142 2.3.3 Microextraction by packed sorbent

143 First MEPS C18 cartridge was activated with 100 µL of MeOH and then further conditioned with 1 mL of
144 H_2O. Then 100 µL of plasma diluted with 400 µL of H_2O were loaded into the cartridge. After the cleaning
145 of the sample with 100 µL of H_2O, 500 µL of MeOH were used for elution. The guide provided by the
146 manufacturers (SGE Analytical Science, Victoria, Australia) was followed for this protocol. Finally, the
147 eluate was dried under N_2 stream at 40 ºC and reconstituted in 100 µL of MeOH:H_2O 50:50 (v:v).

148 2.3.4 Hybrid SPE procedure

149 The protocol performed was the one recommended by the manufacturers (Sigma-Aldrich, St. Louis, MO,
150 USA). Firstly a protein precipitation step was carried out by the addition of 750 µL of cold 1 % FA in ACN
151 to 250 µL of plasma. After a short vortexing the mixture was centrifuged at 1600 g for 3 min. Then 800 µL
152 of the supernatant were loaded into the Hybrid SPE cartridge and vacuum was applied. At this point the
153 eluate is ready for immediate LC-MS/MS analysis, but in order to make this method comparable with the
154 rest of the protocols, it was dried under N_2 stream at 40 ºC and reconstituted in 200 µL of MeOH:H_2O 50:50
155 (v:v).

156 2.4 UHPLC-ESI-TOF-MS analysis

Analysis was performed using an Acquity UPLC System coupled to a electrospray ionization quadrupole-time-of-flight (Q-TOF) Synapt-G2 mass spectrometer (UHPLC-ESI-Q-TOF-MS) (Waters, Milford, MA, USA), operated in positive electrospray ionization mode (ESI +). Chromatography was carried out at 40 ºC on a Waters Acquity BEH C18 column (1.7 µm, 2.1 x 100 mm) with the following solvent system: A = 0.1 % FA in H_2O and B = 0.1 % FA in MeOH. A linear gradient was used at a flow rate of 0.5 mL/min from 100 % A to 100 % B in 21 min. Then 100 % B was hold for 2 min, after this time initial conditions were reached, and finally, a 3 min re-equilibration step was included. The injection volume was 5 µL. ESI conditions were source temperature 120 ºC, desolvation temperature 450 ºC, cone gas flow 10 L/h, desolvation gas flow 900 L/h, capillary voltage 0.7 kV and cone voltage 30 V. TOF detector worked in Resolution mode (approximately 2000 FWHM) and all mass spectral data were acquired in centroid mode by scanning a m/z range of 50-1200 with a scan time of 0.1 s and an interscan delay time of 0.02 s. The quadrupole was not used in this study. Leucine enkephalin ($[M+H]^+$ = 556.2771 m/z) (2 ng/µL in 0.1 % FA in ACN:H_2O 50:50 v:v) was employed as a lock mass, infused straight into the MS at a flow rate of 10 µL/min. The instrument was calibrated before the analysis using a 0.5 mM sodium formate solution (calibration error < 1 ppm). All UHPLC-ESI-TOF-MS operations were run under the control of MassLynx 4.1 (Waters, Milford, MA, USA) software.

2.5 Design of the analysis

A blank, consisted of MeOH:H_2O 50:50 (v:v) solution, was included at the beginning and at the end of the run in order to test any possible contamination or carryover effect. A quality control sample (QC), prepared by combining equal aliquots of the replicates from each method, was injected regularly every 8 injections throughout the run to monitor the sensitivity and the stability of the UHPLC-ESI-TOF-MS platform. This QC sample was also used to condition the system at the beginning of the analysis (QCcond). It was observed in a previous test that at least 15 injections of a sample containing the studied matrix were necessary to stabilize the system and that even after the analysis of a few number of plasma samples the sensitivity fell (data not shown). For this reason and bearing in mind the purpose of the study (evaluating the repeatability of each of the sample preparation protocols), the order of injection of the samples was not randomized to minimize the effect of the instrumental drift arising from column degradation or contamination of the MS

source on the evaluation of repeatability within each protocol. In Table 1 the analysis order is shown. The preparation replicates of each protocol were injected one after the other. Instrumental replicates of one of the preparation replicate were injected between them (Replicate 1_1, 1_2 and 1_3). Although this way of analyzing the samples is the most convenient to study the repeatability, it is not the optimum to set the number of extracted features taking into consideration the drop of sensitivity throughout the run. This problem was overcome analyzing in a different batch 3 replicates of each protocol randomly (data not shown).

2.6 Data processing

The raw spectrometric data acquired were processed using XCMS software [27] (version 1.30.3) in order to convert the three-dimensional LC-MS raw data (RT, m/z, intensity) into a table of time-aligned detected features, with their RT, m/z and intensity in each sample. XCMS is written in R statistical programming language and is freely available under an open-source license. The version of R used was 2.14.1. The samples were grouped according to the different sample preparation protocols. Blanks and QC samples were treated as separate groups. "CentWave" algorithm [28] was used for peak picking with a peak width window of 3-20 s (peakwidth=c(3,20)) and a maximum tolerated m/z deviation in consecutive scans for a peak of 15 ppm (ppm = 15). The m/z width for the grouping was set to 0.015 Da (mzwid = 0.015) and the bandwidth parameter chosen was 5 s (bw = 5) for the first grouping and then determined from the time deviation profile after retention time correction. These values are the commonly used in UHPLC coupled to a high resolution mass spectrometer and studying the raw data and the results obtained it was decided that they were appropriate for the analysis carried out. The rest of the parameters were set to the default values. Finally the missed peaks during the peak picking algorithm were integrated automatically with the "fillPeak" function. This step is essential for reducing the observed CV values, as many zero values are replaced with real peak intensities. In order to avoid LC-MS artifacts (those peaks that do not represent molecular ions of metabolites such as isotopes, common adducts and fragments, multiple charge states, etc), R-package CAMERA was used to filter the detected peaks. The generated peak marker tables (comprising pairs m/z-RT and their corresponding intensity values for each sample) were exported into Microsoft Office Excel 2007 (Redmond, WA, USA) for univariate analysis and into SIMCA-P+ 11.5

(Umetrics, Umea, Sweden) for multivariate analysis. Data out of the interval 0.5-21 min, that is, up to the elution peak and from the point at which column washing step of the analysis started, were discarded.

2.7 Evaluation of method repeatability

In order to obtain the most in-depth information of sample preparation repeatability, data were analyzed using two different approaches, univariate and multivariate statistics. Furthermore, other parameters calculated directly from the resulting table from XCMS, as the total number of features and the coefficient of variation of the area of some selected features for each protocol, can be used to study the repeatability of each protocol.

2.7.1 Multivariate statistics

Principal components analysis (PCA) was performed on all data after logarithmic transformation (10log(peak area)) and mean centering. Scores plots were examined to assess the degree of similarity between the different protocols and to identify outliers or trends in data. The ED within the PCA data was calculated for the intrareplicate distance within the model [29] for each protocol taking into account the first three principal components. These ED values give a comparison of relative repeatability among the replicates, which can be difficult to observe in the obtained 3D PCA score. The lowest this value is the better repeatability it indicates.

2.7.2 Univariate statistics

Univariate statistics were used to compare the CV distribution of feature intensity among the replicates for each protocol. Another way of comparing the CV distribution is calculating the percentage (or number) of features which have a CV lower than a certain value. In this work the number of features with a CV value lower than 0.15, 0.20 and 0.30 were calculated. These two first values are the CV values considered to represent an acceptable degree of repeatability according to the International Conference on Harmonisation (ICH) [30] for bioanalytical methods for targeted analysis in any concentration (0.15), except for the one corresponding to the limit of quantitation (0.20) and the latter (0.30) the acceptable CV value in biomarkers analysis [17].

236 2.8 Other parameters

237 In order to select the most suitable sample preparation protocol for metabolic profiling there are some other
238 parameters that also should be considered. These are: the total number of features obtained and the area
239 deviation of some selected features for each protocol. As the behavior of each detected feature depends on
240 its own chemical structure and the RT at which it elutes from the chromatographic column, no internal
241 labeled standards are commonly used in metabolomics. For this reason the area deviation offered by each
242 protocol was assessed by studying six features with different RTs and m/z present in all the replicates of all
243 the protocols.

244 2.9 Protein concentration estimation

245 The Bradford assay was employed to estimate the amount of protein remaining in three replicates of each
246 reconstituted plasma preparation protocol. Samples were first diluted 1:2 (v:v) with distilled H_2O and then
247 mixed with Bradford reagent in a ratio of 1:20 (v:v) sample:Bradford reagent. A calibration curve was
248 generated using known concentrations (from 0.78 to 100 mg/L) of a standard protein (BSA).

249 3 Results

250 The 8 sample preparation protocols (each performed on 6 preparation replicates and 3 instrumental
251 replicates) were compared resulting in a set of 90 injections, 64 plasma samples plus 24 QC samples (QC
252 + QC conditioning) and 2 blanks. Different criteria were used to evaluate the protocols: repeatability by
253 multivariate and univariate analyses, number of extracted features, repeatability of a series of selected
254 features and the amount of protein remaining in each reconstituted plasma extract.

255 3.1 Feature distribution

256 The base peak intensity (BPI) chromatograms obtained with all the sample preparation protocols show the
257 feature distribution along chromatographic time(Fig 2). In general, until the minute 16 all of them, except
258 Hybrid SPE and MEPS had similar profiles with different intensities and number of visible features,
259 especially higher for ACN 2:1 and MeOH 2:1. From this minute on, in the cases of Hybrid SPE, MEPS,

direct SPE and SPE + PPT, fewer chromatographic peaks were detected comparing with the rest of protocols, and again ACN 2:1 and MeOH 2:1 provided the highest peak intensities and number of features. Taking into account that the last minutes correspond to the lipophilic region we could conclude that solvent precipitation extracts had more lipophilic material than other protocols and that the removal of a great part of this kind of compounds was achieved when any cartridge was used for sample preparation.

3.2 Evaluation of method repeatability using multivariate statistics

The repeatability of the chromatographic method used was assessed in the PCA. A clear tendency of the QCs is observed as the instrument sensitivity falls (44 % of signal drop from the first QC to the last one, data not shown). The explained variation (R^2X) was 70.5, 79.9 and 86.7 for the first, second and third principal components, respectively, and the predicted variation (Q^2X) for these components was 70.2, 79.5 and 86.1. When the model was built without including the QCs samples in the data processing step (Fig 3), in order to avoid their effect in the PCA, a high increase in R^2X and Q^2X parameter was observed for the first three components (91.6, 96.0 and 97.9 and 88.5, 92.6 and 95.8, respectively). All the protocols were well separated from each other and the results agreed with the differences observed in the BPI chromatograms. This can be observed in the distribution of the sample treatment protocols along the first principal component.

In order to give a number to the relative repeatability of the protocols shown by PCA scores plot, the ED values generated from the first three components were calculated. In Fig 4, the ED values represent the mean of the distances among the replicates of a particular protocol.

ACN 2:1 protocol yielded the lowest mean ED value. When this value was statistically compared (95 % confidence level) with the ED values of the rest of sample preparation protocols significant differences were obtained in all cases, except for SPE + PPT .

3.3 Evaluation of method repeatability using univariate statistics

Box plots were used to visualize the distribution of feature intensity CV values among the replicates (Fig 5). Each box shows the degree of dispersion of CV values of features for one condition by displaying the

285 25^{th} percentile (bottom of the box), the median, the mean and the 75^{th} percentile (top of the box). According
286 to this plot, the protocols based on solvent precipitation with ACN in a 2:1 organic solvent:plasma ratio,
287 showing a median and mean CV of 0.12 and 0.15, respectively, was the most repeatable protocol. An
288 analysis of variance confirmed that the observed differences between protocols were significant.

289 Another way of visualizing the distribution of feature intensity CV values is calculating the number of
290 extracted features for each method with an intensity CV lower than 0.15, 0.20 and 0.30. These data are
291 shown in Fig 6. In terms of number of consistently detected features, again ACN 2:1 protocol seemed to be
292 the most repeatable protocol, having the 93 % of the features a CV below 0.30. It is important to bear in
293 mind that the values of the total number of features indicated in this section is not real because it is affected
294 by the fall of sensitivity during the analysis.

295 3.4 Number of extracted features

296 The number of extracted features from XCMS for each protocol was also considered in the selection of
297 optimal conditions. As it was mentioned in section 2.5 this value was calculated injecting in a different
298 batch only 3 replicates from each protocol randomly, so that the drop of sensitivity along a long run does
299 not lead to an underestimation of the number of features of the samples analyzed at the end of the batch
300 (data not shown). The obtained order, in terms of number of extracted features, did not differ from the one
301 observed in the designed run. As Table 2 shows, the maximum number of detected features was achieved
302 with PPT with MeOH 2:1 as protocol closely followed by ACN 2:1. The best feature extraction efficiency
303 of MeOH in serum samples was reported by Want et al [15] and in plasma samples by Bruce et al [16]. A
304 high decrease in the number of extracted features was observed for Hybrid SPE, MEPS and direct SPE,
305 which makes sense taking into account that the former includes a mechanism for the removal of
306 phospholipids and in C18 cartridges based protocols without a previous protein precipitation step protein
307 bound compounds are not studied, while protocols including a protein precipitation step result in a drastic
308 alteration of the 3D structure of proteins that allows the release of metabolites bound to them.

309 The number of common repeatable features between methods was calculated. Pairwise comparisons of the
310 different plasma preparation protocols showing the number of common features and its percentage taking

as 100 % the total detected features in the protocol with less total detected features in each case are indicated in Table 2. A high percentage of common features (> 76 %) can be seen for the different proportions of organic solvent:plasma used, higher in the case of ACN (87 %). On the other hand, it is observed that using solid phase extraction protocols it is possible to detect features that are not observed in PPT protocols, meaning that both types of protocol are complementary.

3.5 Repeatability of selected metabolites

Analytical and sample preparation repeatability was further investigated using selected features. For this purpose 6 features with different RTs and m/z present in all the replicates of all the protocols were chosen. Table 3 lists these features and their measured m/z value, RTs, and intensity CV among the replicates for each protocol.

All the protocols provided a mean CV value < 0.2, yielding the best results in terms of repeatability SPE + PPT, offering a mean CV value of 0.12 among preparation replicates and 0.04 when only instrumental replicates are taken into account.

3.6 Protein concentration

The concentration of residual protein was estimated for each plasma preparation protocol using Bradford assay. According to the results obtained by Bruce et al [16] ACN offers a better protein removal efficiency than MeOH in plasma samples. As it can be observed in Table 4,our results agree with these observations being both protein precipitations protocols based on PPT with MeOH the ones that contained the largest amount of residual protein. The efficiency of SPE cartridges in terms of protein removal has not been reported before in any of the aforementioned studies, but attending to these results, the use of these cartridges helps to get a cleaner extract compared with a simple PPT with MeOH, as it was expected. It is worthwhile to remark that in the case of SPE + PPT protocol, MeOH was used as organic solvent for PPT and, therefore, that maybe if ACN had been used as protein precipitation solvent this combination would have got the lowest remaining protein concentration.

4 Discussion

Many reported approaches for plasma sample preparation have focused on a small subset of metabolites [14, 16-18]. As our aim was to perform untargeted metabolic profiling of plasma samples encompassing a wide range of chemical structures, it was also essential to study the repeatability of the different plasma preparation protocols used for all the detected features, as it was reported by Want et al [15]. For this reason efficiency and repeatability of the selected plasma preparation protocols were investigated using univariate and multivariate analyses and other parameters, such as feature distribution, number of extracted features, the repeatability of some selected features and the amount of protein remaining in the reconstituted samples, were studied. All of these criteria should be taken into account for selecting a sample preparation protocol for metabolic profiling studies.

To our knowledge only in one work the evaluation of the use of SPE cartridges in metabolomics has been carried out [17], but authors did not study sample preparation repeatability. On the other hand, it is the first time in which the efficiency of SPE + PPT, MEPS and Hybrid SPE in metabolomics is evaluated.

Before starting to analyze the different studied plasma preparation protocols it is worth pointing out that an appropriate design of the study is essential to fulfill the desired aim. The applied LC-MS method entails a drop of sensitivity along a single run (44 %) and this fact needs to be taken into consideration, otherwise the results would not be reliable at all. The proposed design to solve this problem is to inject the samples, instead of randomly, classified by protocols. In this way the highest drop of sensitivity within a protocol is only of 7 % (calculated from the instrumental replicates) and the distance from the first replicate to the last one is constant for all the protocols. Another point to bear in mind related to the drop of sensitivity is its effect on the number of detected features parameter. To overcome this, a different analysis in which only 3 preparation replicates of each protocol are analyzed randomly is carried out.

After the evaluation of the different criteria applied, we found that PPT with ACN in a 2:1 (v/v) ratio with plasma produced the best results for most of the parameters studied. This protocol offered one of the lowest EDs between replicates in multivariate analysis, the lowest CV values in univariate analysis, good repeatability of the selected features, and one of the lowest concentrations of remaining protein in the final extract. Furthermore, although PPT with ACN in a 2:1 ratio with plasma offered 9.5 % fewer total detected

features than PPT with MeOH in a 2:1 ratio with plasma, the difference in terms of repeatable features (CV<0.30) was only 1.2 %. The satisfactory results in terms of repeatability could be explained by the combination of the simplicity of the sample preparation protocol itself (the same for all the PPT protocols) and the high efficiency of ACN as a PPT agent (the HybridSPE protocol also included a PPT with ACN step). The better results achieved by PPTwith ACN when compared with MeOHin terms of repeatability could be explained by the fact that the extracts obtained with PPT using ACN as an organic solvent exhibited lower ionization suppression than the ones obtained with MeOH [31]. On the other hand, in the comparison of PPTwith ACN with the HybridSPE protocol it must be taken into account that the HybridSPE protocol includes an additional step that could be the cause of the observed differences in repeatability. The lower number of extracted features of the protocols that include retention mechanisms was probably due, on the one hand, in the case of direct SPE and MEPS, to the fact that metabolites bound to proteins are not released and, on the other hand, when HybridSPE cartridges were used, to the removal of phospholipids. Finally, according to the Bradford assay, the extracts obtained using ACN as an agent for PPTwere cleaner than the ones obtained with MeOH, which were the extracts that contained the highest concentration of remaining proteins. This agrees with the results reported by Bruce et al. [16], and thus, MeOH should not be an option when a long column life is desired.

Although MichopoulosAlthough Michopoulos et al [17] observed that SPE offered good repeatability, our results do not totally agree when it is compared with PPT protocols. A reason for this difference could be that they did not include in their work preparation replicates and that they only focused on a small subset of metabolites. Another fact that differs from their study and ours is that all our protocols were carried out manually and they used an automatized SPE station, which could significantly improve repeatability. Want et al [15] set that ACN was the organic solvent which provided less repeatability in terms of univariate analysis in serum samples. The reason for the difference between their results and ours cannot be easily established, but could be because serum and plasma are similar, but different matrices. Another explanation could be that they injected the sample preparation replicates randomly, and therefore, sample treatments with replicates further from each other show a higher CV owing to the decay in sensitivity.

The MEPS and HybridSPE protocols did not result in the expected good repeatability, but maybe the automatization of the processes and/or the optimization of the parameters that affect the extractions could significantly improve the results.

In terms of complementarity it is important to mention that pairwise comparison showed that many of the detected features in protocols that included solid phase extraction mechanism were not detected when only PPT protocols were used. These findings mean that combaining both types of protocols a higher coverage of the metabolome could be obtained.

Finally, this work shows an approach to compare the results obtained from different sample preparation protocols for untargeted metabolomics that takes into consideration many different parameters that affect directly the results. This strategy could be applied to any other matrix and/or sample preparation protocols.

5 Conclusions

We have reported an appropriate design of a study for the evaluation of different human plasma sample preparation protocols for use in a metabolomic study. After the evaluation of the eight plasma preparation protocols studied (based on organic solvent precipitation or three different cartridges or a combination of both), we can conclude that a simple PPT with ACN in a 2:1 (v/v) ratio with plasma is the protocol that globally better satisfies all the requirements established: the best results in terms of univariate and multivariate repeatability, a high number of extracted features, and good removal of proteins from the sample, which is very relevant when a great number of samples is to be analyzed. On the other hand, the use of PPT- and cartridge-based extraction protocols offers a different coverage of the metabolome.

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7 References

[1] Nicholson JK, Lindon JC, Holmes E (1999) 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. Xenobiotica 29:1181-1189

[2] Goodacre R, Vaidyanathan S, Dunn WB, Harrigan GG, Kell DB (2004) Metabolomics by numbers: acquiring and understanding global metabolite data. Trends Biotechnol 22:245-252

[3] Fiehn O, Kopka J, Dormann P, Altmann T, Trethewey RN, Willmitzer L, (2000) Metabolite profiling for plant functional genomics. Nat Biotechnol 18:1157-1161

[4] Van der Greef J, Stroobant P, Van der Heijden R (2004) The role of analytical sciences in medical systems biology. Curr Opin Chem Biol 8:559-565

[5] Lindon JC, Nicholson JK, Holmes E (2007) The Handbook of Metabonomics and Metabolomics. Elsevier, Amsterdam

[6] Griffiths WJ (2008) Metabolomics, metabonomics and metabolite profiling. The Royal Society of Chemistry, Cambridge

[7] Lenz EM, Wilson ID (2007) Analytical strategies in metabolomics. J Proteome Res 6:443-458.

[8] Pan Z, Raftery D (2007) Comparing and combining NMR spectroscopy and mass spectrometry in metabolomics. Anal Bioanal Chem 387:525-527

[9] Viswanathan CT, Bansal S, Booth B, DeStefano AJ, Rose MJ, Sailstad J, Shah VP, Skelly JP, Swann PG, Weiner R (2007) Quantitative bioanalytical methods validation and implementation: Best practices for chromatographic and ligand binding assays. Pharm Res 24:1962-1973

433 [10] Gika HG, Theodoridis GA, Wingate JE, Wilson ID (2007) Within-day reproducibility of an LC-MS-
434 based method for metabonomic analysis: application to human urine. J Proteome Res 6(8):3291-303

435 [11] Masson P, Alves AC, Ebbels TM, Nicholson JK, Want EJ (2010) Optimization and evaluation of
436 metabolite extraction protocols for untargeted metabolic profiling of liver samples by UPLC-MS. Anal
437 Chem 82(18):7779-7786

438 [12] Pan L, Qiu Y, Chen T, Lin J, Chi Y, Su M, Zhao A, Jia W (2010) An optimized procedure for
439 metabonomic analysis of rat liver tissue using gas chromatography/time-of-flight mass spectrometry. J
440 Pharm Biomed 52(4):589-596

441 [13] Jiye A, Trygg J, Gullberg J, Johansson AI, Jonsson P, Antti H, Marklund SL, Moritz T (2005) Extraction
442 and GC/MS analysis of the human blood plasma metabolome. Anal Chem 77:8086-8094

443 [14] Bruce SJ, Jonsson P, Antti H, Cioarec O, Trygg J, Marklund SL, Moritz T (2008) Evaluation of a
444 protocol for metabolic profiling studies on human blood plasma by combined UPLC-MS: from extraction
445 to data analysis. Anal Biochem 372:237-249

446 [15] Want EJ, O'Maille G, Smith CA, Brandon TR, Uritboonthai W, Qin C, Trauger SA, Siuzdak G. (2006)
447 Solvent-dependent metabolite distribution, clustering, and protein extraction for serum profiling with mass
448 spectrometry. Anal Chem 78:743-752

449 [16] Bruce SJ, Tavazzi I, Parisod V, Rezzi S, Kochhar S, Guy PA (2009) Investigation of human blood
450 plasma sample preparation for performing metabolomics using ultrahigh performance liquid
451 chromatography/mass spectrometry. Anal Chem 81:3285-3296

452 [17] Michopoulos F, Lai L, Gika HG, Theodoridis G, Wilson ID (2009) UPLC-MS-based analysis of
453 human plasma for metabolomics using solvent precipitation or solid phase extraction. J Proteome Res,
454 8:2114-2121

[18] Tulipani S, Llorach R, Urpi-Sarda M, Andres-Lacueva C (2013) Comparative analysis of sample preparation methods to handle the complexity of the blood fluid metabolome: when less is more. Anal Chem 85(1):341-348

[19] Marcinowska R, Trygg J, Wolf-Watz H, Mortiz T, Surowiec I (2011) Optimization of a sample preparation method for the metabolomic analysis of clinically relevant bacteria. J Microbiol Methods 87(1):24-31

[20] Madla S, Miura D, Wariishi H (2012) Optimization of extraction method for GC-MS based metabolomics for *filamentous fungi*. J Microbial Biochem Technol 4(1):5-9

[21] Sellick CA, Knight D, Croxford AS, Maqsood AR, Stephens GM, Goodacre R, Dickson AJ (2010) Evaluation of extraction processes for intracellular metabolite profiling of mammalian cells: matching extraction approaches to cell type and metabolite targets. Metabolomics 6(3):427-438

[22] Dettmer K, Nurnberger N, Kaspar H, Gruber MA, Almstetter MF, Oefner PJ (2011) Metabolite extraction from adherently growing mammalian cells for metabolomics studies: optimization of harvesting and extraction protocols. Anal Bioanal Chem, 399(3):1127-1139

[23] Neubauer S, Haberhauer-Troyer C, Klavins K, Russmayer H, Steiger MG, Gasser B, Sauer M, Mattanovich D, Hann S, Koellensperger G (2012) U13C cell extract of *Pichia pastoris* - a powerful tool for evaluation of sample preparation in metabolomics. J Sep Sci 35:3091-3105

[24] Kim S, Lee DY, Wohlgemuth G, Park HS, Fiehn, O, Kim H (2013) Evaluation and optimization of metabolome sample preparation methods for *Saccharomyces cerevisiae*. Anal Chem 85(4):2169-2176

[25] Wu J, An Y, Yao J, Wang Y, Tang H (2010) An optimised sample preparation method for NMR-based faecal metabonomic analysis. Analyst 135:1023-1030

476 [26] Jiang H, Zhang Y, Ida M, LaFayette A, Fast DM (2011) Determination of carboplatin in human plasma
477 using HybridSPE-precipitation along with liquid chromatography-tandem mass spectrometry. J
478 Chromatogr B 879(22):2162-2170

479 [27] Smith CA, Want EJ, O'Maille G, Abagyan R, Siuzdak G (2006) XCMS: processing mass spectrometry
480 data for metabolite profiling using nonlinear peak alignment, matching, and identification. Anal Chem
481 78:779-787

482 [28] Tautenhahn R, Böttcher C, Neumann, S (2008) Highly sensitive feature detection for high resolution
483 LC/MS. BMC Bioinformatics, 9:504-519

484 [29] Guy PA, Tavazzi I, Bruce SJ, Ramadan Z, Kochhar SJ (2008) Global metabolic profiling analysis on
485 human urine by UPLC-TOF-MS: Issues and method validation in nutritional metabolomics. J Chromatogr B
486 871:253-260

487 [30] ICH Expert Working Work. ICH Harmonised tripartite guideline, validation of analytical procedures:
488 Text and methodology Q2 (R1) (2005) Geneva

489 [31] Polson C, Sarkar P, Incledon B, Raguvaran V, Grant R (2003) Optimization of protein precipitation
490 based upon effectiveness of protein removal and ionization effect in liquid chromatography-tandem mass
491 spectrometry. J Chromatogr B 785:263-275

492

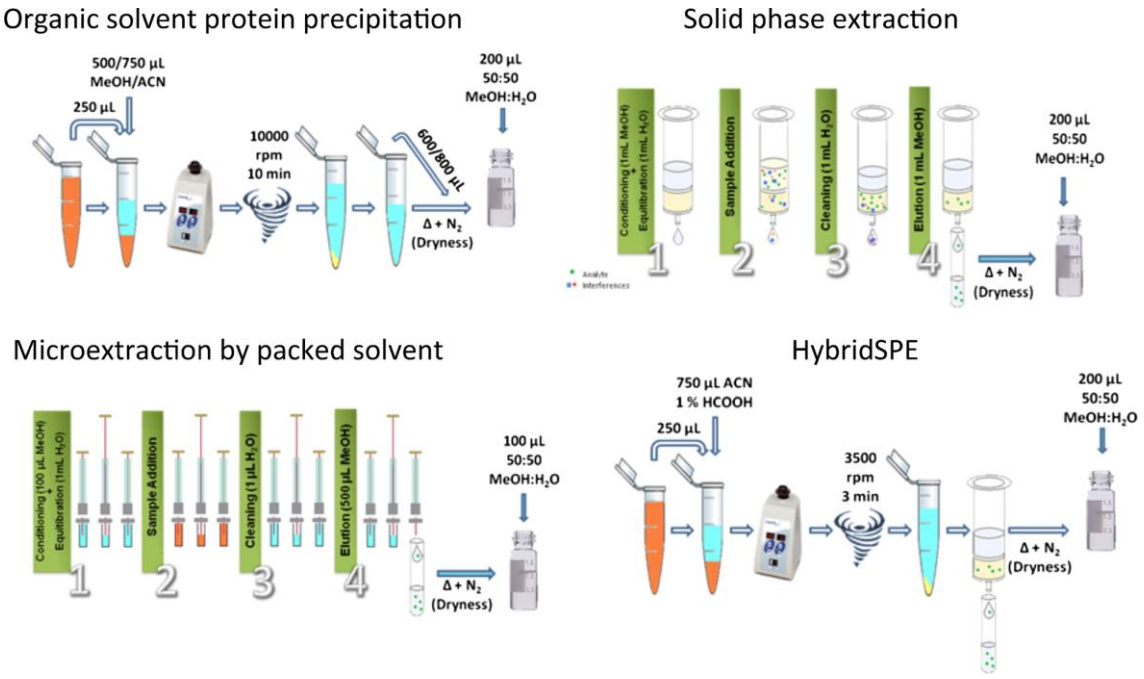

Fig. 1 Sample treatment protocols evaluated. ACN acetonitrile, MeOH methanol

Table 1 Order of sample injection

Order of injection of the different protocols	Injection schedule within each protocol
15 QC conditioning	QC
MEPS	Replicate 1_1
SPE	Replicate 2
HybridSPE	Replicate 3
SPE + PPT	Replicate 1_2
MeOH 2:1	Replicate 4
ACN 2:1	Replicate 5
MeOH 3:1	Replicate 6
ACN 3:1	Replicate 1_3
	QC

ACN 2:1 acetonitrile in a 2:1 (v/v) ratio with plasma, *ACN 3:1* acetonitrile in a 3:1 (v/v) ratio with plasma, *MEPS* microextraction by packed sorbent, *MeOH 2:1* methanol in a 2:1 (v/v) ratio with plasma, *MeOH 3:1* methanol in a 3:1 (v/v) ratio with plasma, *PPT* protein precipitation, *QC* quality control, *SPE* solid-phase extraction

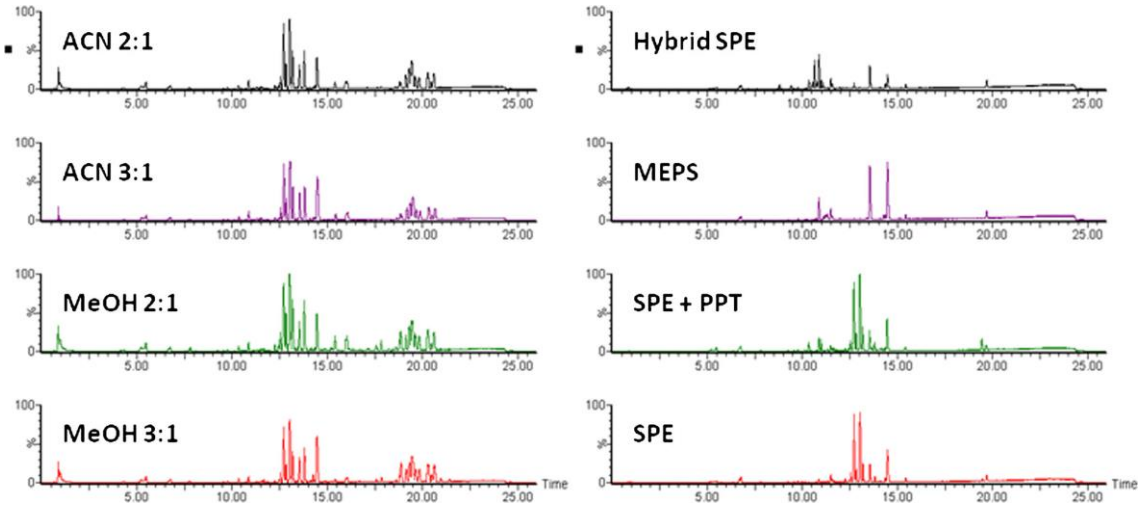

Fig. 2 Raw base peak intensity ultra-high-performance liquid chromatography (UHPLC)–electrospray ionization-time-of-flight mass spectrometry chromatograms of a pooled plasma sample treated with the eight preparation protocols—ACN in a 2:1 (v/v) ratio with plasma (ACN 2:1), ACN in a 3:1 (v/v) ratio with plasma (ACN 3:1), MeOH in a 2:1 (v/v) ratio with plasma (MeOH 2:1), MeOH in a 3:1 (v/v) ratio with plasma (MeOH 3:1), HybridSPE, microextraction by packed sorbent (MEPS), solid phase extraction (SPE) and protein precipitation (PPT), and direct SPE—injected through a BEH C_{18} HPLC column (2.1 mm×100 mm).

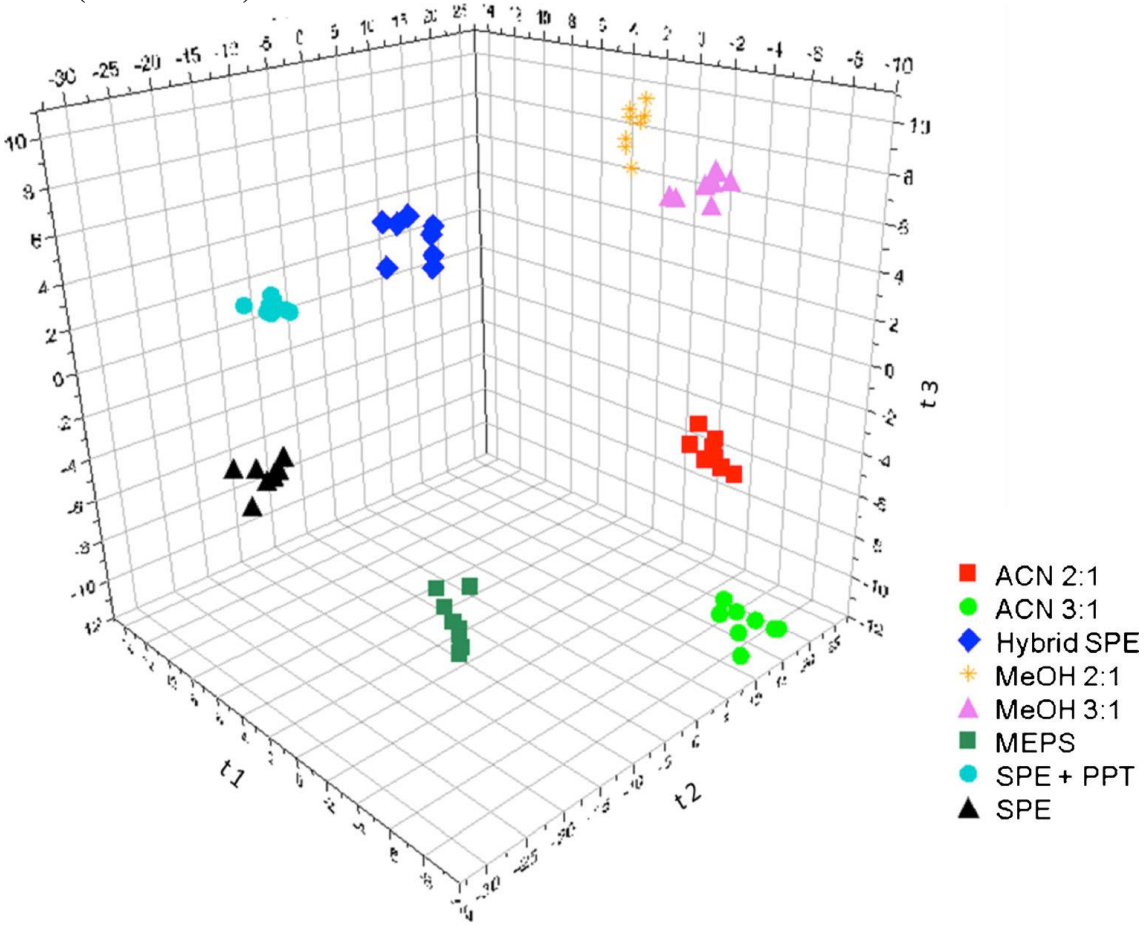

Fig. 3 Three-dimensional principal component analysis scores plot for all the plasma preparation protocols after removal of liquid chromatography–mass spectrometry artifacts and quality control samples

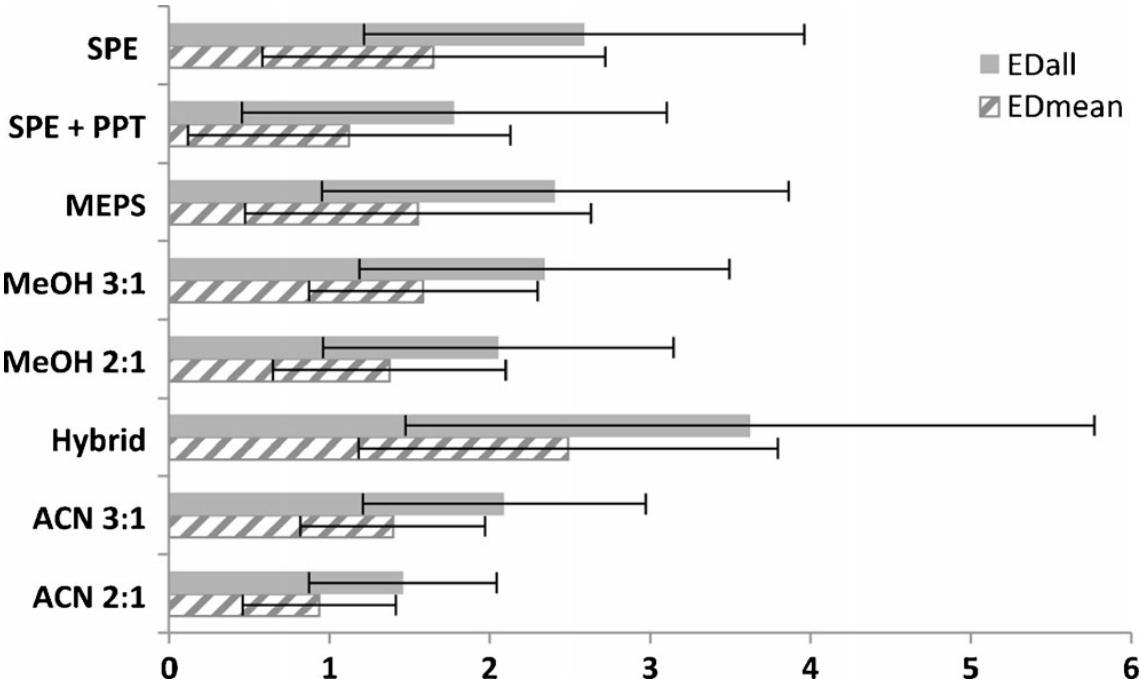

Fig. 4 Calculated Euclidean distances (ED) (expressed as mean±confidence interval (95 % confidence level)

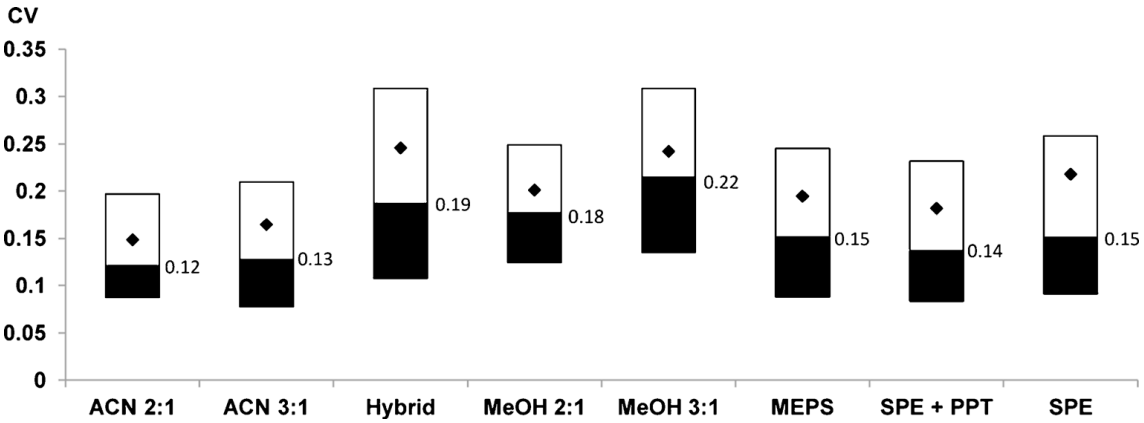

Fig. 5 Box plots comparing the distribution of feature intensity coefficients of variation (CV) among replicates for the different sample preparation protocols. For each box, the bottom corresponds to the 25th percentile, the middle band corresponds to the median (numerical value indicated), the diamond corresponds to the mean, and the top corresponds to the 75th percentile

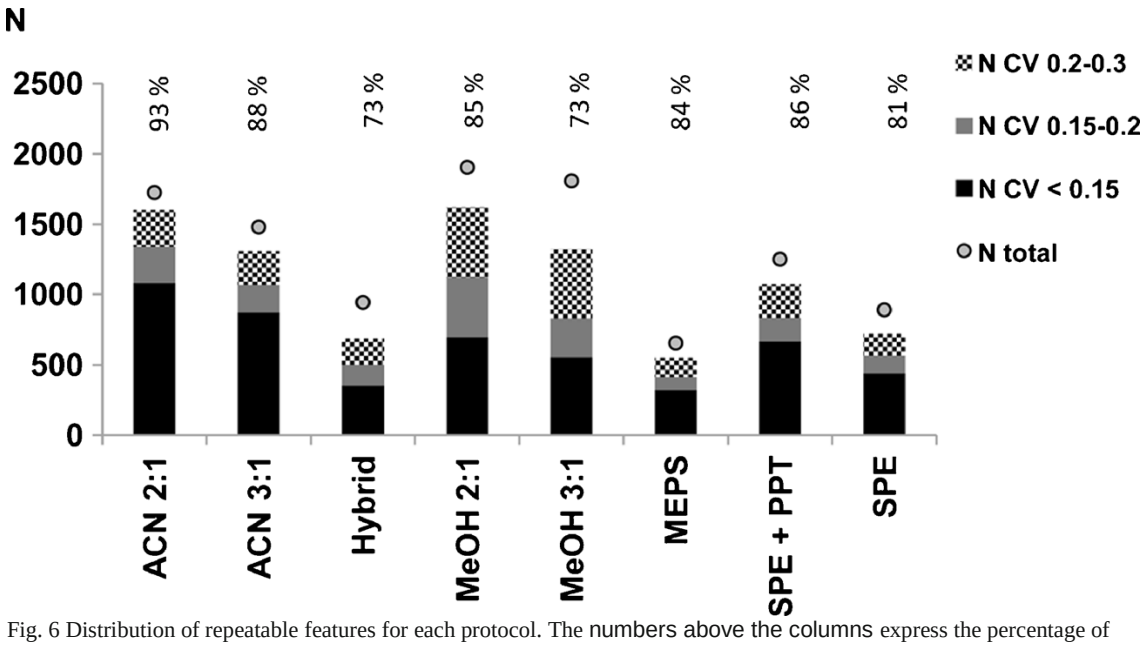

Fig. 6 Distribution of repeatable features for each protocol. The numbers above the columns express the percentage of features with a CV<0.30 of the total number of detected features

Table 2 Pairwise comparison showing the number of common features and the percentage, taking as 100 % the total detected features in the protocol, and the total detected features in each case

	ACN 2:1	ACN 3:1	HybridSPE	MeOH 2:1	MeOH 3:1	MEPS	SPE + PPT	SPE	Total
SPE	592 (67)	533 (60)	494 (56)	566 (64)	496 (58)	423 (64)	730 (82)		890
SPE + PPT	796 (64)	719 (58)	545 (58)	846 (68)	732 (59)	436 (66)			1,250
MEPS	351 (54)	324 (49)	479 (73)	353 (54)	298 (45)				656
MeOH 3:1	1201 (70)	1109 (75)	347 (37)	1366 (76)					1,808
MeOH 2:1	1289 (75)	1158 (78)	429 (45)						1,905
HybridSPE	420 (45)	363 (38)							943
ACN 3:1	1287 (87)								1,480
Total	1,725	1,480	943	1,905	1,808	656	1,250	890	

Table 3 Repeatability of six features with different *m/z* and retention times (*RT*) present in all the replicates of each sample preparation protocol

Feature	*m/z*	RT (s)	CV							
			ACN 2:1	ACN 3:1	HybridSPE	MeOH 2:1	MeOH 3:1	MEPS	SPE + PPT	SPE
M227T46	226.952	45.8	0.06	0.15	0.09	0.11	0.10	0.27	0.04	0.22
M195T406	195.088	405.6	0.15	0.23	0.23	0.20	0.03	0.10	0.21	0.11
M314T587	314.233	587.0	0.11	0.06	0.29	0.16	0.13	0.14	0.08	0.19
M460T652	460.270	652.3	0.21	0.18	0.12	0.22	0.57	0.10	0.18	0.48
M637T772	637.306	772.3	0.27	0.24	0.25	0.18	0.20	0.11	0.15	0.10
M804T862	803.543	862.3	0.05	0.07	0.03	0.09	0.13	0.06	0.06	0.12
Mean CV			0.14	0.15	0.17	0.16	0.19	0.13	0.12	0.20
Mean CV of instrumental replicates			0.11	0.16	0.05	0.18	0.11	0.07	0.04	0.17

CV coefficient of variation

Table 4 Absorbance measurements and concentrations of residual protein (expressed as mean±confidence interval; 95 % confidence level) estimated for each plasma preparation protocol using the Bradford assay

Bradford assay	ACN 2:1	ACN 3:1	HybridSPE	MeOH 2:1	MeOH 3:1	MEPS	SPE + PPT	SPE
Measure 1	0.625	0.598	0.586	1.619	1.559	0.635	0.680	0.735
Measure 2	0.603	0.599	0.605	1.614	1.49	0.590	0.734	0.777
Measure 3	0.622	0.589	0.596	1.664	1.499	0.622	0.695	0.658
Mean	0.617	0.595	0.596	1.632	1.516	0.635	0.703	0.723
Concentration (mg/L)	2.5±0.4	1.9±0.2	2.0±0.4	26±1	24±2	3±1	4±1	5±3

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