Journal of Chromatography B, 898 (2012) 111–120

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Intracellular metabolite profiling of platelets: Evaluation of extraction processes and chromatographic strategies

Giuseppe Paglia[a], Manuela Magnúsdóttir[a], Steinunn Thorlacius[a], Ólafur E. Sigurjónsson[b,c], Sveinn Guðmundsson[b], Bernhard Ø. Palsson[a], Ines Thiele[a,d,*]

[a] Center for Systems Biology, University of Iceland, Sturlugata 8, Reykjavik, Iceland
[b] The Blood Bank, Landspitali-University Hospital, Snorrabraut 60, Reykjavik, Iceland
[c] School of Science and Engineering, Reykjavik University, Menntavegur 1, Reykjavik, Iceland
[d] Faculty of Industrial Engineering, Mechanical Engineering & Computer Science, University of Iceland, Reykjavik, Iceland

ARTICLE INFO

Article history:
Received 24 February 2012
Accepted 22 April 2012
Available online 30 April 2012

Keywords:
Metabolomics
UPLC–MS
Platelets
Extraction
HILIC
Reversed phase liquid chromatography

ABSTRACT

An extraction method for intracellular metabolite profiling should ideally be able to recover the broadest possible range of metabolites present in a sample. However, the development of such methods is hampered by the diversity of the physico-chemical properties of metabolites as well as by the specific characteristics of samples and cells. In this study, we report the optimization of an UPLC–MS method for the metabolite analysis of platelet samples. The optimal analytical protocol was determined by testing seven different extraction methods as well as by employing two different LC–MS methods, in which the metabolites were separated by using hydrophilic interaction liquid chromatography (HILIC) and reversed phase liquid chromatography (RPLC). The optimal conditions were selected using the coverage of the platelets' metabolome, the response of the identified metabolites, the reproducibility of the analytical method, and the time of the analysis as main evaluation criteria. Our results show that methanol–water (7:3) extraction coupled with HILIC–MS method provides the best compromise, allowing identification of 107 metabolites in a platelet cell extract sample, 91% of them with a RSD% lower than 20. A higher number of metabolites could be detected when analyzing the platelet samples with two different LC–MS methods or when using complementary extraction methods in parallel.

1. Introduction

Metabolomics is a comparative qualitative and quantitative analysis of low-molecular weight metabolites in a given biological system. As an emerging field, it has produced exciting achievements in biomedical science in recent years [1–5]. Metabolic profiling provides a global picture of the metabolome by maximizing the number of detected metabolites without a priori knowledge of the metabolites present in a sample [6,7]. Quantification and identification of metabolites extracted from biological complex matrices, such as serum, urine, and cells require sophisticated analytical instrumentation, i.e., mass spectrometry (MS) and nuclear magnetic resonance (NMR) [8–12].

MS provides specific chemical information, which is directly related to the chemical structure of a compound, such as accurate mass, isotope distribution patterns, and characteristic fragment ions, aiding structure elucidation [13]. Often, MS is preceded by a chromatographic separation in order to reduce matrix effect and ionization suppression as well as to enable separation of isomeric and isobaric compounds; thus, allowing more accurate identification and quantification of metabolites. Gas chromatography (GC), liquid chromatography (LC), and capillary electrophoresis (CE) are usually employed to separate metabolites in complex samples prior to the MS analysis [9,14–16].

Typically, an untargeted LC/MS metabolomics workflow consists of cell quenching and metabolite extraction followed by appropriate separation protocols. Ideally, an extraction method should be able to quantitatively recover the broadest possible range of metabolites. However, due to differences in chemical mass, physico-chemical properties, and a very large dynamic range of concentration ranges, this remains a difficult task to achieve. Moreover, extraction protocols are sample- and cell-dependent and different extraction methods have been recommended for mammalian cells. For instance, different methods have been reported for metabolite extraction from human fibroblast cells [17], Madin-Darby canine kidney cells [18], Chinese hamster ovary (CHO) cells [19–21], and erythrocytes [22,23].

To date, there are no metabolomic studies reporting analytical protocols for platelets. Platelets are small anucleate cells having

* Corresponding author at: Center for Systems Biology, University of Iceland, Sturlugata 8, Reykjavik, Iceland. Tel.: +354 6186246.
E-mail addresses: ithiele@hi.is, ines.thiele@gmail.com (I. Thiele).

1570-0232/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jchromb.2012.04.026

an important role in thrombosis, hemostasis, inflammation, and wound healing [24,25]. Platelets are stored and used in transfusion medicine; hence, the understanding of changes occurring during their storage, termed platelets storage lesions, has recently gained importance [26]. For many years, it was thought that the majority of platelets proteins were synthesized by megakaryocytes and acquired from them during their maturation. Recent evidence has shown that de novo protein synthesis occurs in mature platelets and that it plays a role in platelets storage lesions process [26]. Consequently, platelets appear to be metabolically more active than previously thought.

In this study, we report the evaluation of different extraction methods in order to achieve the largest possible coverage of the platelet metabolome. Moreover, since a metabolomic approach often employs different analytical platforms to maximize the number of metabolites detected [27], extracted samples were analyzed using hydrophilic interaction liquid chromatography (HILIC) and reversed phase liquid chromatography (RPLC) coupled to MS in order to obtain the best analytical approach for high throughput intracellular measurements of platelet samples. Based on our chief criteria, which included the number and amount of metabolites recovered as well as reproducibility and time, we found that methanol–water extraction coupled with HILIC–MS provides the best analytical workflow for platelets.

2. Material and methods

2.1. Chemicals

All materials were obtained from Sigma–Aldrich (Seelze, Germany) unless stated otherwise. Acetonitrile was purchased from Merck (Darmstadt, Germany). Water was obtained using an 18 Ωm Milli-Q (Millipore, USA). All chemicals and solvents were of analytical grade or higher purity.

2.2. Platelet samples

A platelet unit was obtained from the Blood Bank (The Blood Bank, Landspitali-University Hospital, Reykjavik, Iceland). The unit derived from five blood donors and platelets were obtained with the buffy coat method and stored for 5 days in standard conditions (22 °C under gentle agitation) in a T-Sol solution containing sodium citrate (2.94 g), sodium acetate (4.08 g), and sodium chloride (6.75 g) in 1000 mL of H_2O at pH 7.2, more 10–20% of plasma coming from the blood donors. The study has been approved by the National Bioethics Committee of Iceland and The Icelandic Data Protection Authority.

2.3. Extraction methods

In all extractions, 0.5 mL of sample was collected (8×10^8 cells mL^{-1}). Cells were isolated by centrifuging for 5 min at 1500 × *g*. Cell metabolism was quenched adding cold or hot methanol, as described for each extraction method. For each extraction procedure, four independent extractions were performed.

2.3.1. Methanol water (pH 7) extraction

0.5 mL of methanol–water (7:3) at the temperature of −20 °C were added to the cell pellets and samples were vortexed for 1 min. Cell lysis was achieved by performing two consecutive freeze and thaw steps. Samples were centrifuged for 15 min at 15,000 × *g* and 3 μL of the supernatant were directly injected in the UPLC–MS system during both HILIC and RPLC experiments.

2.3.2. Methanol:water acidic (pH 2) extraction

A solution of methanol–water (7:3) was prepared using water containing 1% of formic acid (pH 2). 0.5 mL of this mixture at the temperature of −20 °C were added to the cell pellets. Samples were vortexed for 1 min and cell lysis was achieved by performing two consecutively freeze and thaw steps. Samples were centrifuged for 15 min at 15,000 × *g* and 3 μL of the supernatant were directly injected in the UPLC–MS system during both HILIC and RPLC experiments.

2.3.3. Methanol:water basic (pH 10) extraction

A solution of methanol–water (7:3) was prepared using water containing 2% of sodium hydroxide (pH 10). 0.5 mL of this mixture at the temperature of −20 °C were added to the cell pellets. Samples were vortexed for 1 min and cell lysis was achieved by performing two consecutively freeze and thaw steps. Samples were centrifuged for 15 min at 15,000 × *g* and 3 μL of the supernatant were directly injected in the UPLC–MS system both during HILIC and RPLC experiments.

2.3.4. Hot methanol (80 °C) extraction

The cell pellets were resuspended in 0.5 mL of methanol at the temperature of 80 °C and incubated for 15 min at 80 °C. Samples were cooled down in ice for 10 min and vortexed for 1 min before being centrifuged for 15 min at 15,000 × *g*. 3 μL of the supernatant were directly injected in the UPLC–MS system during both HILIC and RPLC experiments.

2.3.5. Methanol:acetonitrile:water (ACN) extraction

0.5 mL of methanol:acetonitrile:water (4:4:2) at the temperature of −20 °C were added to the cell pellets. Samples were vortexed for 1 min and cell lysis was achieved by performing two consecutively freeze and thaw steps. Samples were centrifuged for 15 min at 15,000 × *g* and 3 μL of the supernatant were directly injected in the UPLC–MS system during both HILIC and RPLC experiments.

2.3.6. Methanol:chloroform:water ($CHCl_3$) extraction

Cell pellets were resuspended in 1.2 mL of methanol at the temperature of −20 °C. Samples were vortexed for 1 min and cell lysis was achieved by performing two consecutively freeze and thaw steps. 0.6 mL of chloroform was added to the samples and vortexed for 30 s during a period of 15 min maintaining the sample in cold bath. 0.2 mL of ice-cold water was added to the sample and vortexed for 1 min. The tubes were centrifuged for 1 min at 1000 × *g* and transferred to the freezer at −20 °C for 4 h. The organic and water phase were recovered, pooled together, and dried under gentle stream of nitrogen. Dried samples were reconstituted in 0.5 mL of methanol:water (7:3) and centrifuged for 15 min at 15,000 × *g* to precipitate residual proteins. 3 μL of the supernatant were injected in the UPLC–MS system during both HILIC and RPLC experiments.

2.3.7. Methanol + water two-step (two-step) extraction

1.4 mL of methanol at the temperature of −20 °C were added to the cell pellets. Samples were vortexed for 1 min and cell lysis was achieved by performing two consecutively freeze and thaw steps. Samples were centrifuged for 5 min at 1500 × *g*. The supernatant was collected in another tube. A second step extraction was achieved adding 0.6 mL of ice-cold water and samples were vortexed for 1 min. Samples were centrifuged for 15 min at 15,000 × *g* and the water extracts were added to the methanol ones. Samples were dried under gentle stream of nitrogen and reconstituted in 0.5 mL of methanol:water (7:3). 3 μL were injected in the UPLC–MS system during both HILIC and RPLC experiments.

2.4. UPLC–MS

All analyses were performed with an UPLC system (UPLC Acquity, Waters, Manchester, UK) coupled in line with a quadrupole-time of flight hybrid mass spectrometer (Synapt G2, Waters, Manchester, UK). An electrospray ionization interface was used to direct column eluent to the mass spectrometer. The chromatographic separation was achieved using both HILIC and RPLC columns, as described in Sections 2.4.1 and 2.4.2.

2.4.1. Hydrophilic interaction liquid chromatography

For HILIC analysis an Acquity amide column, 1.7 μm (2.1 mm × 150 mm) (Waters, Manchester, UK) was used. The flow rate, in both negative and positive mode, was 0.4 mL/min. In positive mode, mobile phase A (100% acetonitrile) and B (100% water) both containing 0.1% formic acid, and the following elution gradient was used: 0 min 99% A; 8 min 20% A; 8.5 min 99% A; 10 min 99% A. For analysis in negative mode, mobile phase A contained acetonitrile:sodium bicarbonate 10 mM (95:5) and mobile phase B contained acetonitrile:sodium bicarbonate 10 mM (5:95). The following elution gradient was used: 0 min 99% A; 2 min 80% A; 5 min 20% A; 6 min 20% A; 6.5 min 99% A; 10 min 99% A.

2.4.2. Reversed phase liquid chromatography

For RPLC analysis, an Acquity HSS T3 column, 1.8 μm (2.1 mm × 150 mm) (Waters, Manchester, UK) was used. The same conditions were used in both negative and positive mode. The flow rate was 0.4 mL/min with mobile phase A (methanol) and mobile phase B (water), both containing 0.1% formic acid. The elution gradient was: 0 min 99% B; 5.5 min 1% B; 8 min 1% B; 8.2 min 99% B; 10 min 99% B.

2.4.3. Mass spectrometry

The mass spectrometer operated in V mode for high sensitivity using a capillary voltage of 1.8 kV and a cone voltage of 25 V. Cone and desolvation gas flow was 20 and 800 L/h, respectively, while source and desolvation gas temperature was 120 and 500 °C, respectively. MS spectra were acquired in centroid mode from m/z 50 to 1000 using scan time of 0.5 s. Leucine enkephalin (2 ng/μL) was used as lock mass (m/z 556.2771 and 554.2615 in positive and negative experiments, respectively).

2.5. Data processing and metabolites identification

MarkerLynx (v4.1, Waters) was used to integrate and align MS data points and to convert them into exact mass retention time pairs (EMRT). Multivariate statistical analysis was performed applying EZinfo (Umetrics) using EMRT pairs. Principal component analysis (PCA) was performed using pareto scaling on all detected features.

QuanLynx (v4.1, Waters) was used to integrate chromatograms of tentatively identified metabolites. Extracted ion chromatograms (EICs) were extracted using a 0.02 mDa window centered on the expected m/z for each targeted compound.

The identity of the peaks was established by verifying peak retention time, accurate mass measurements, and tandem mass spectrometry against our in-house database and/or online databases, including HMDB (http://www.hmdb.ca/) and METLIN (http://metlin.scripps.edu/). Only metabolites identified in all 4 replicates of each extraction were considered.

3. Results and discussion

The primary focus of this work was the evaluation of extraction processes and chromatographic strategies for analysis of intracellular metabolites of platelets, in order to develop a fast and reliable UPLC–MS method for studying metabolic changes occurring in platelets during their storage in transfusion medicine. To address this challenge, we compared extracted platelet metabolites obtained from seven different extraction methods and analyzed the extracted samples with HILIC– and RPLC–MS methods.

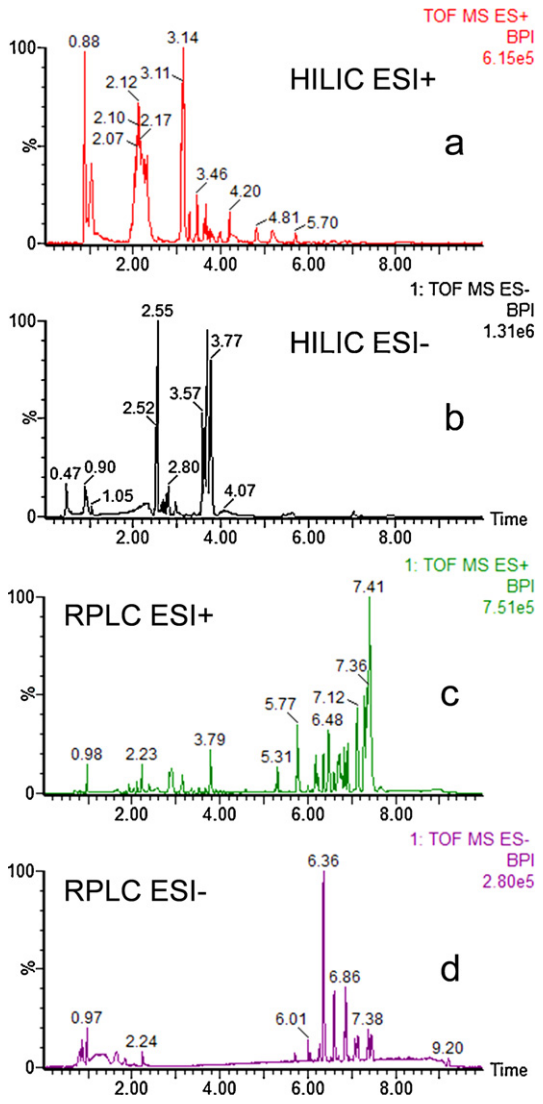

Fig. 1. Base peak ion chromatograms of platelets extracted with methanol:water (7:3) at pH 7. The *x*-axis represents time in minutes, while the *y*-axis indicated the relative intensity. (a) HILIC in positive mode. (b) HILIC in negative mode. (c) RPLC in positive mode. (d) RPLC in negative mode.

Methanol–water extraction has been reported to be the best method for metabolite profiling of CHO cells [19], while a liquid–liquid extraction (LLE) (methanol:chloroform:water) has been recommended at pH 2 and/or pH 9 in order to have better coverage of the erythrocytes metabolome [22]. Based on these reports, a methanol–water extraction at neutral pH was selected as main method and other six procedures were developed by modifying the main method using acidic (pH 2) and alkali conditions (pH 10), hot methanol (80 °C), a mixture methanol:acetonitrile:water (ACN), a LLE with chloroform ($CHCl_3$), and a two step process with methanol + water (two-step) (see also Section 2). The extracted samples were successively analyzed using two different UPLC–MS approaches, i.e., HILIC and RPLC. The obtained results from each experimental condition were evaluated considering quantitative and qualitative response of the identified metabolites,

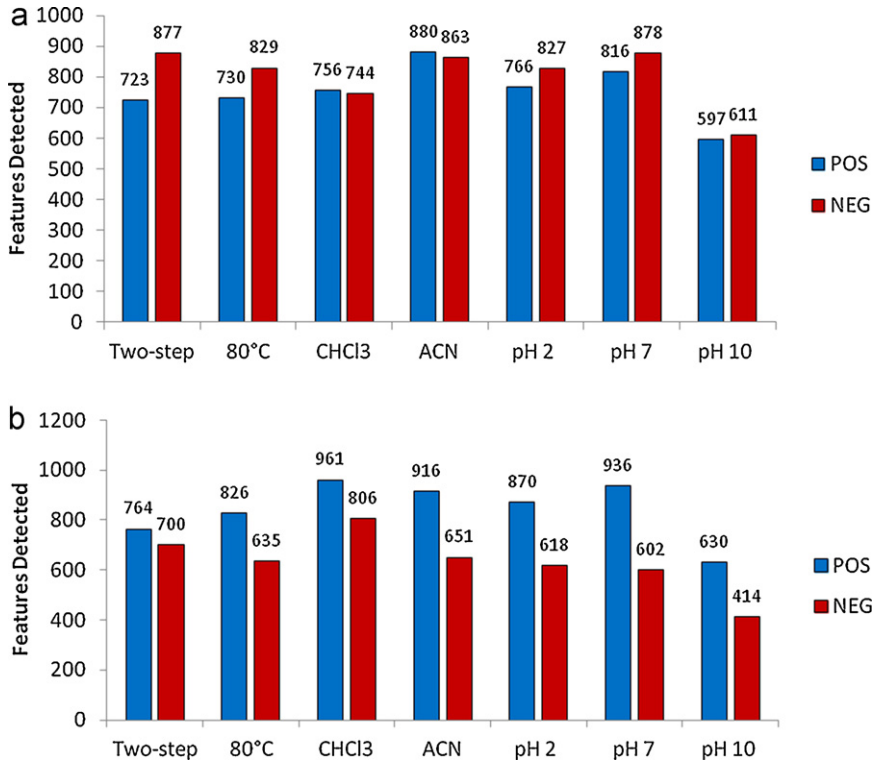

Fig. 2. Number of features detected. (a) HILIC, and (b) RPLC. Features were obtained by integrating and aligning the MS data. After normalization for the sum of all signals the data were converted into exact mass retention time pairs.

the reproducibility, and the suitability of these procedures for high throughput analysis.

3.1. Evaluation of extraction procedures using HILIC–MS method

Water is the major constituent of cells, so it is reasonable to speculate that a high number of polar metabolites might be present in their intracellular content. It has been demonstrated that HILIC–MS methods easily retain and resolve polar metabolites and that those are suitable methods for their identification and quantification [28,29]. Therefore, we decided to employ this chromatographic approach for intracellular measurements of platelet extracts. Typical HILIC–MS profiles in positive and negative mode are shown in Fig. 1a and b.

The number of features detected in positive mode ranged from 880 (ACN) to 597 (pH 10), while in negative mode they ranged from

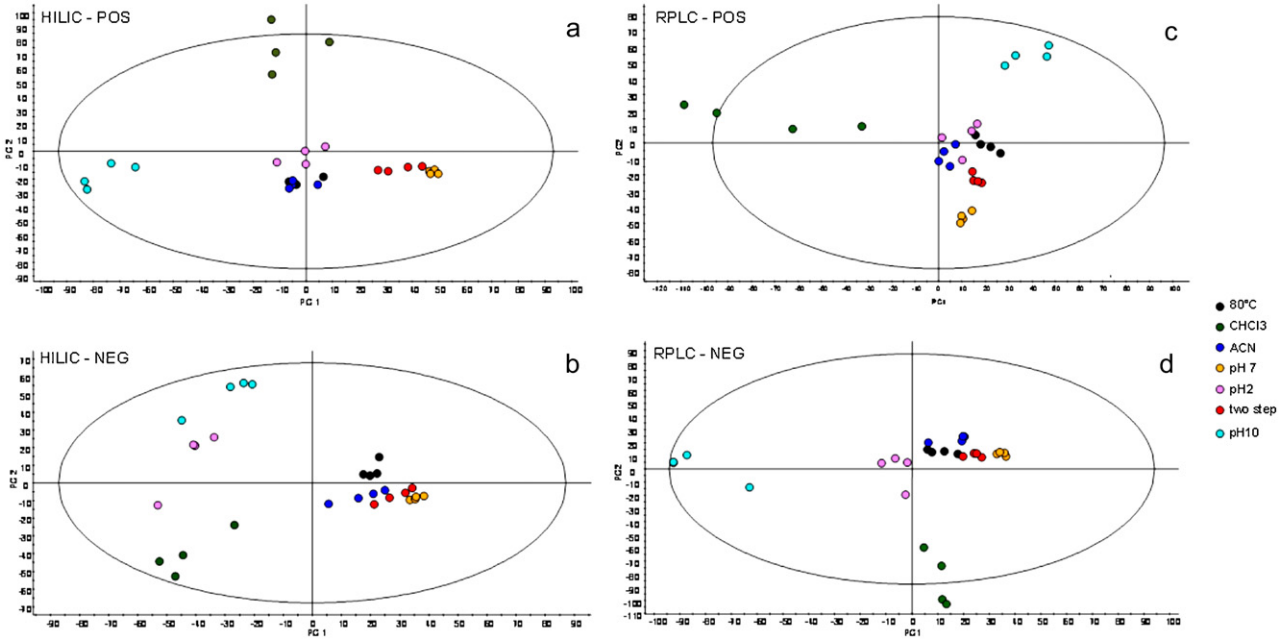

Fig. 3. Principal component analysis (PCA). (a) HILIC–MS in positive mode. (b) HILIC–MS in negative mode. (c) RPLC–MS in positive mode. (d) RPLC–MS in negative mode. All detected features were considered for PCA by using pareto scaling.

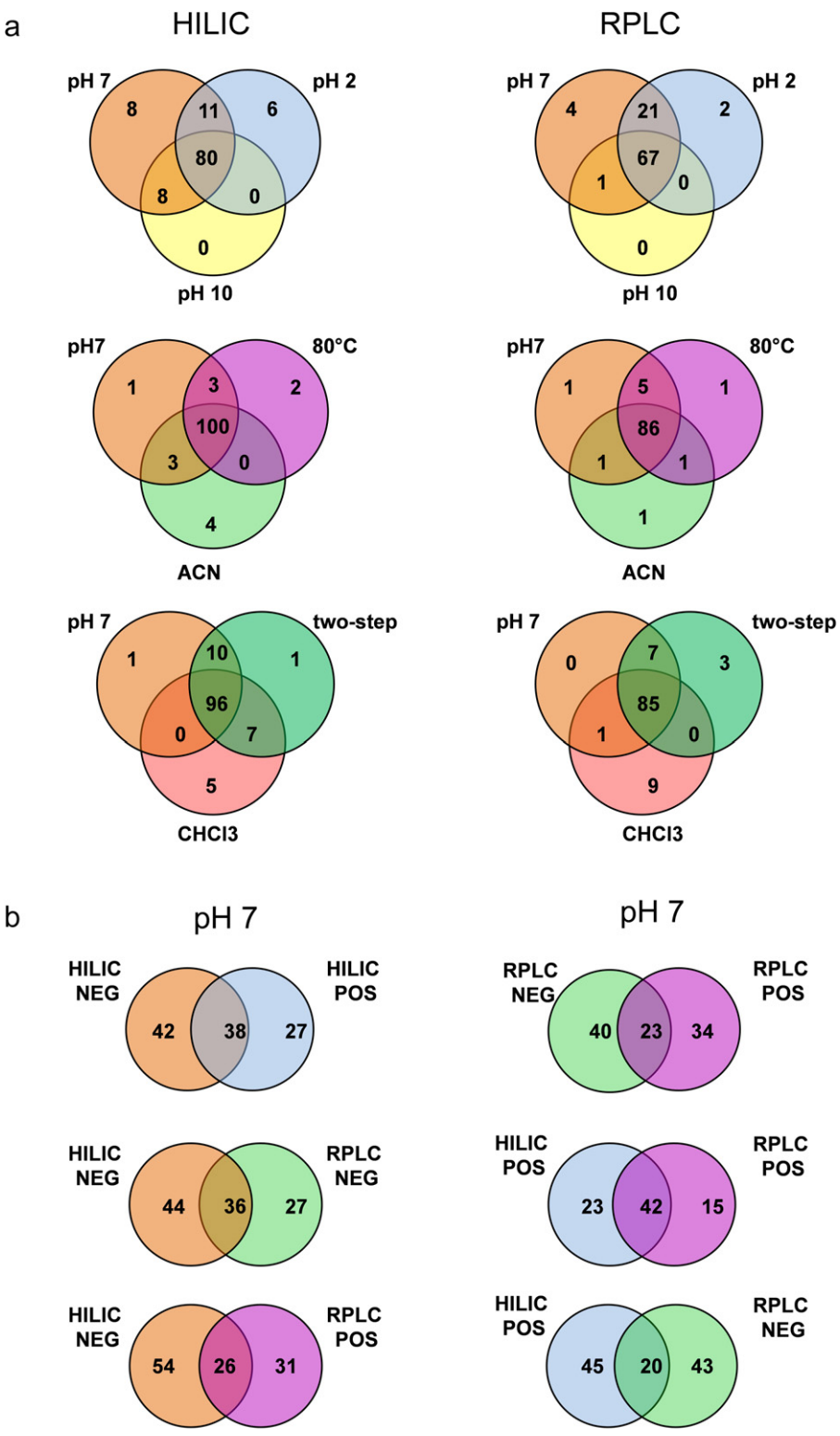

Fig. 4. Shared metabolites in different experimental conditions. (a) Venn diagrams highlighting the identified metabolites that were shared between the different extraction methods during HILIC– and RPLC–MS. The methanol:water (7:3) extraction at neutral pH (pH 7) was used as benchmark for each case. (b) Venn diagrams highlighting overlap between identified metabolites in positive and negative ionization.

878 (pH 7) to 611 (pH 10) (Fig. 2). Based on this first evidence, it is possible to say that extractions employing basic conditions recovered less metabolites compared to the other extractions, which did not show significant differences in the number of detected features. These findings were confirmed when the dataset was visualized using PCA showing that pH 10 and $CHCl_3$ extractions clustered separately, while other extractions clustered closer to each other (Fig. 3a and b).

Even if the number of features detected would provide a useful picture of the metabolome of a particular biological sample, many signals detected by the UPLC–MS system might be due to fragments, adducts, and condensation products, and some signals might

Table 1
Number of metabolites tentatively identified in each experimental condition.

All conditions	151
RPLC all extractions	106
HILIC all extractions	121
In common (HILIC and RPLC)	75
Only with RPLC	31
Only with HILIC	46

Extractions	pH 2	pH 7	pH 10	80 °C	ACN	$CHCl_3$	Two-step	Common to all extractions
RPLC	90	93	68	93	89	95	95	65
HILIC	97	107	88	105	108	108	114	77

also come from background noise. Therefore, we further evaluated metabolites that were tentatively identified during HILIC–MS experiments. Of the 121 metabolites we identified, only 77 were common to all extractions (Table 1). In agreement with the number of features detected, the pH 10 extraction method provided the lowest numbers of recovered metabolites (88), while the highest number (108) was identified using a two-step extraction, confirming that the largest fraction of the metabolome has an affinity to polar solvents (Table 1). On the other hand, with the exception of acidic and basic conditions, there were only few differences in the number of metabolites identified between the other conditions. Fig. 4a shows the distribution of these metabolites between the different conditions. Acidic, basic, and neutral conditions shared 80 metabolites. At pH 10, we did not recover 25 metabolites, while 16 metabolites were not detected in acidic conditions (Fig. 4a). The comparison of methanol–water (7:3) extractions at three different pH conditions demonstrated that, for the platelet samples and using our experimental conditions, neutral pH provided the best condition in terms of the number of recovered metabolites. No significant differences were noticed when comparing extraction performed at pH 7 using either ACN or boiling methanol (80 °C) (Fig. 4a). When we compared the metabolites recovered from pH 7, two-step and $CHCl_3$ extractions, we found that 12 metabolites were not identified when chloroform was used, while at pH 7, 13 metabolites were missing compared to the other two methods (Fig. 4a).

As a next step, we monitored the response of some metabolites (Fig. 5). For example, we observed that amino acids showed,

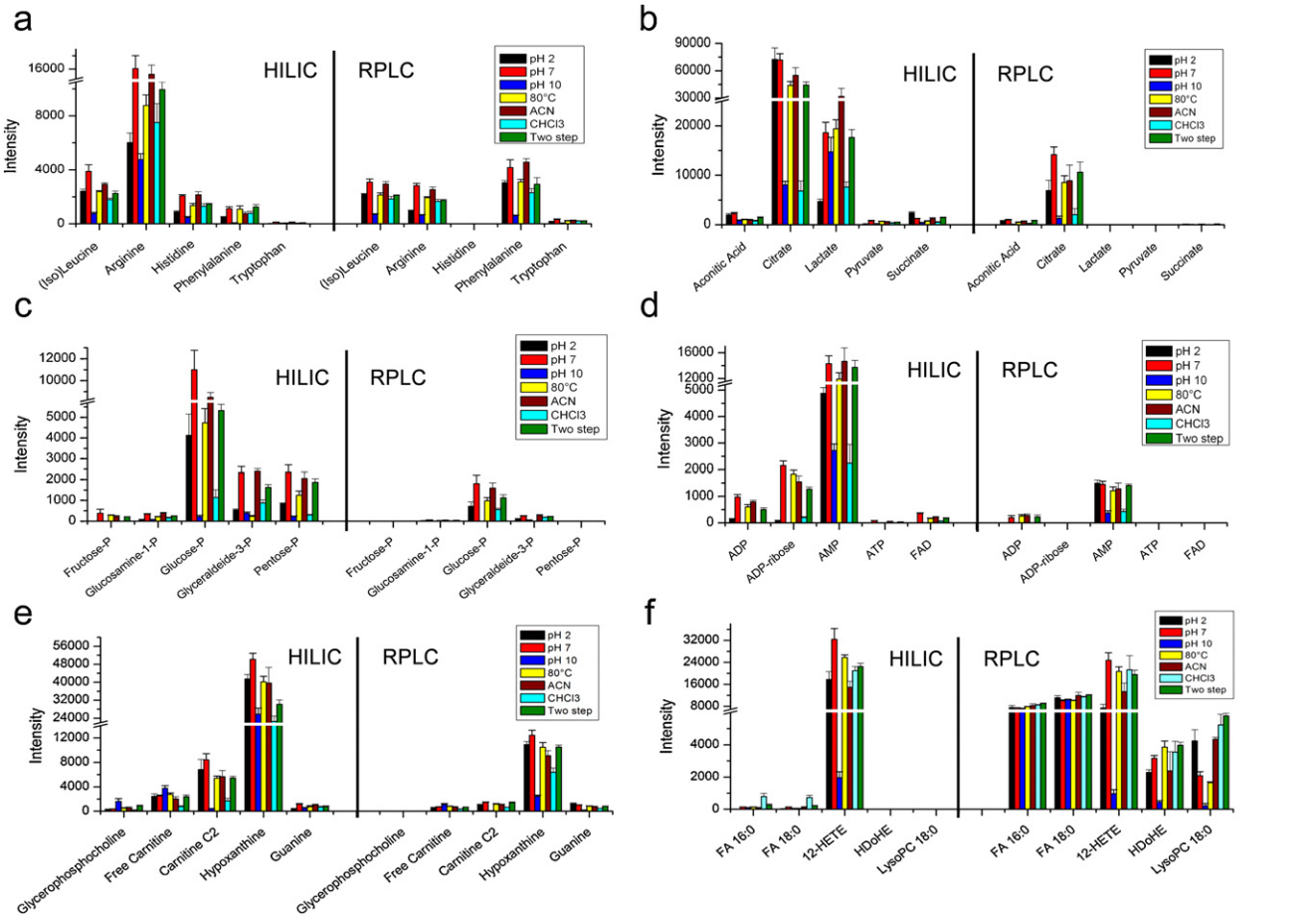

Fig. 5. Chromatographic peak area response of selected metabolites in HILIC– and RPLC–MS when using different extraction methods. Each bar represents the average of four independent extractions. Error bars represent the standard deviations. (a) Amino acids. (b) Organic acids. (c) Sugar phosphates. (d) Phosphorylated metabolites. (e) Selected metabolites. (f) Lipids.

as a general trend, a better response when they were extracted under neutral condition (pH 7) with a two-step or the ACN extraction method (Fig. 5a). The response of organic acids was similar in neutral and acidic conditions, with the exception of lactate and pyruvate, whereas the response was ten times lower when pH 10 or $CHCl_3$ extraction was employed (Fig. 5b). Phosphorylated compounds showed increased response when methanol–water extraction was used at neutral pH. In fact, the response decreased when $CHCl_3$, acidic, or basic conditions were used (Fig. 5c and d), probably due to the degradation of these compounds under these conditions. Some other metabolites, such as free carnitine and glycerophosphocholine, showed an opposite trend as the response increased when we employed basic conditions. These metabolites are zwitterionic compounds and tend to be water soluble at most pH conditions. Therefore, using strong basic conditions may cause degradation of acylcarnitines and choline-containing phospholipids leading to an increased amount of the respective degradation products in the extract. Fatty acids and lipids exhibited, in general, better response when the two-step or $CHCl_3$ extractions were used (Fig. 5e and f), while 12-hydroxyeicosatetraenoic acid (12-HETE) had a better response under neutral conditions.

We also evaluated the relative standard deviation (RSD%) of the identified metabolites in each extraction (Fig. 6a). In the pH 7 extraction, 91% of the metabolites had an RSD% ranging from 0 to 20. The pH 2 and the two-step extractions yielded 80% of the metabolites with a RSD% lower than 20, but they also showed a higher number of metabolites with an RSD% between 10 and 20. The worst RSD% was recorded for pH 10 and $CHCl_3$ extractions, where less than 70% of metabolites had a RSD% below 20. As a general trend, we can say that methanol:water extractions at neutral conditions (pH 7) was the best method when coupled with HILIC–MS analysis.

3.2. Evaluation of extraction procedures using RPLC–MS method

Reversed phase liquid chromatography (RPLC) is probably the most popular strategy adopted in metabolomic studies employing LC–MS systems, which is due to its optimal performances in terms of reproducibility and robustness. Usually, compounds with medium to low polarity are retained and resolved during this chromatographic approach, while highly polar compounds are typically eluted in the void volume of the column. In our RPLC–MS experiments, a HSS T3 column was chosen because it provided a more efficient retention and separation of polar compound compared with other C18 columns tested (data not shown). The chromatographic profiles obtained from the analysis of platelet extracts are shown in Fig. 1c and d. As in HILIC–MS, the lowest amount of features detected was obtained when basic conditions were employed. In fact, a remarkable difference was obtained (30% and 50% less features detected in positive mode and negative mode, respectively) when compared with the number of features detected when using the LLE ($CHCl_3$) (Fig. 2). This difference between pH 10 and $CHCl_3$ was also observed when PCA was performed as the data grouped into two well defined clusters (Fig. 3). When we continued with the metabolites identification, we could only identify 68 metabolites in the basic conditions (Table 1). The number of metabolites detected with $CHCl_3$ was, in contrast, similar to the ones recovered with the remaining conditions. Since the number of metabolites detected does not explain the different clustering of $CHCl_3$ extracted samples in PCA, the reason can be attributed to the identity and to the quantitative response of the detected signals. In fact, nine metabolites, i.e., lipid compounds, were detected in $CHCl_3$ extraction, which were not extracted with methanol–water based methods (Fig. 4a). In contrast, the methanol–water extractions, using one and two-step, recovered ten metabolites, that could not be detected during $CHCl_3$ extraction. A total of 27 metabolites were missing from the

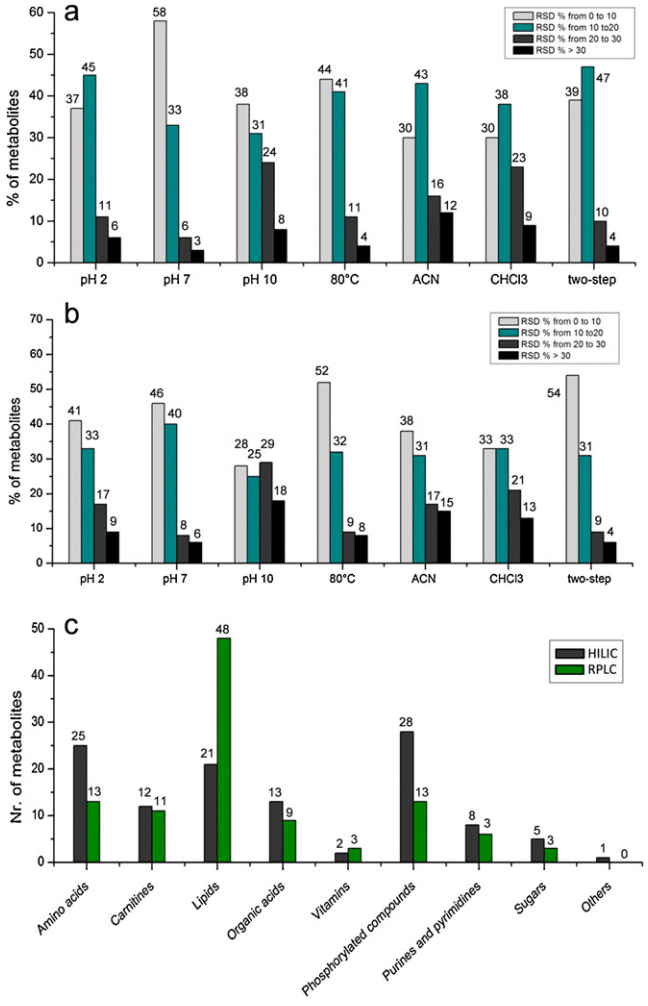

Fig. 6. Reproducibility and identification of metabolites based on the chromatography used. (a) Relative standard deviation (RSD %) for metabolites identified with each extraction method during HILIC–MS analysis. Value is presented as percentage of metabolites. (b) RSD% for metabolites identified with each extraction methods during RPLC–MS analysis. Value is presented as percentage of metabolites. (c) Comparison of metabolites identified with HILIC– and RPLC–MS divided in different classes. Lipid class contains fatty acids, eicosanoids, phospholipids, and sphingolipids. Phosphorylated compounds include nucleotides, phosphorylated sugars, and FAD. Purine and pyrimidines do not include phosphorylated compounds.

alkali extraction when compared with acidic and neutral extractions. Many of these metabolites were phosphorylated compounds and lipids, which were probably degraded during the extraction process.

We found that amino acids were better recovered in the RPLC–MS analysis when using pH 7, even though only amino acids of low polarity, such as phenylalanine and (iso)leucine, showed good sensitivity (Fig. 5a). A similar trend was observed for other polar compounds, such as phosphorylated sugars, nucleotides, and organic acids, which showed generally better response during pH 7 extractions (Fig. 5b–d). In contrast, stearic and palmitic acid showed a similar response in all conditions (Fig. 5f). This behavior was different from the detected eicosanoids. For instance, the response of 12-HETE was 3 and 12 fold lower at pH 2 and 10, respectively. We suspect that acidic and basic conditions induced degradation of lipids (triglycerides, phospholipids, etc.) generating free fatty acids, thus, increasing their response under these conditions and explaining different response between eicosanoids and fatty acids.

Overall, during RPLC analysis, the two-step extraction provided the best reproducibility with 85% of identified metabolites with

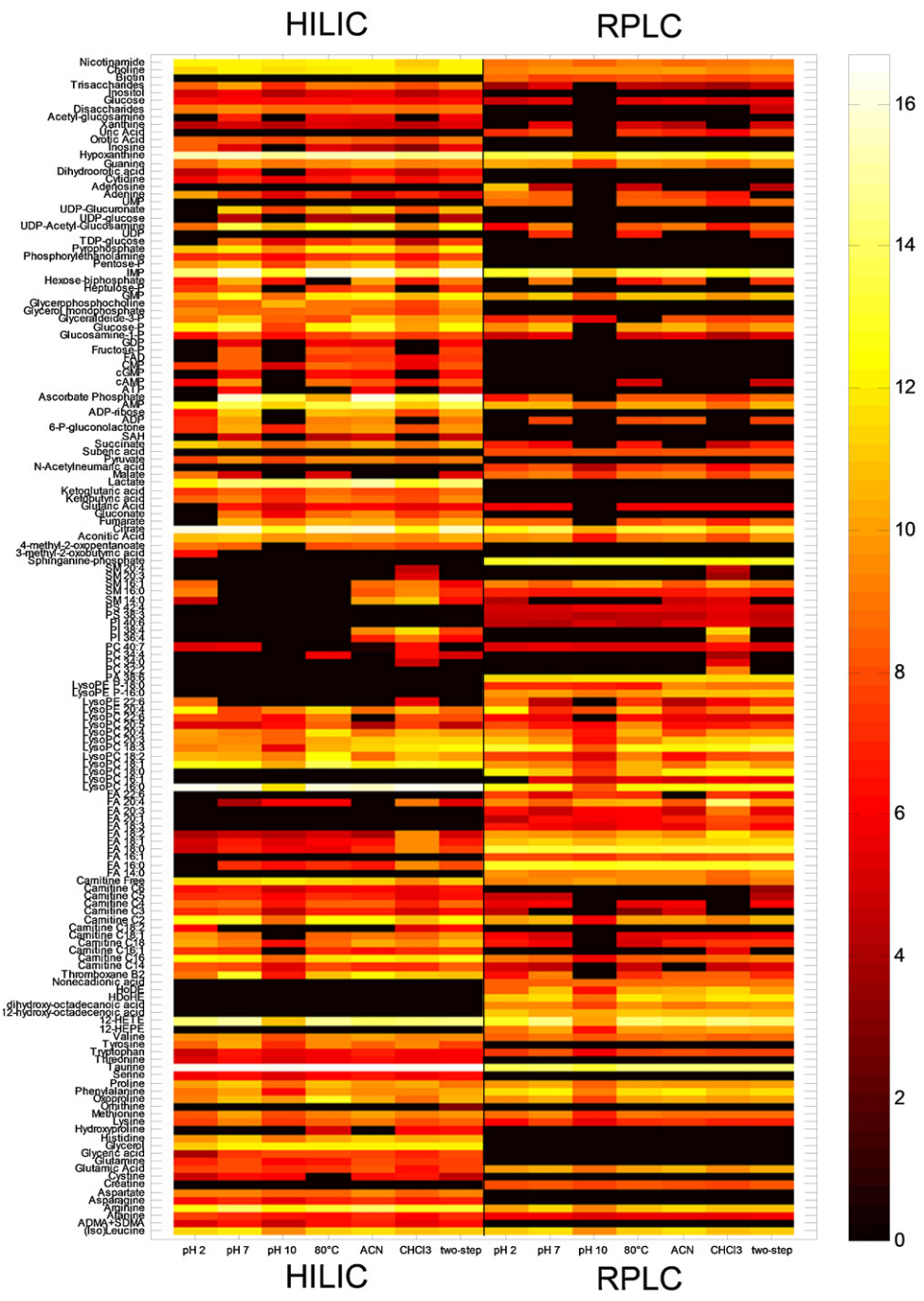

Fig. 7. A heatmap of all tentatively identified metabolites recovered with each extraction method and analyzed with HILIC– and RPLC–MS. Each value represents the average of four independent extractions. Chromatographic peak area response is reported in logarithmic scale.

an RSD% lower than 20 and 54% of metabolites showing an RSD% lower than 10. The $CHCl_3$ and pH 10 conditions resulted in lower reproducibility. The reason may be the more complicated procedure, which requires more than one step, in the case of the $CHCl_3$ extraction.

3.3. Comparison of HILIC and RPLC

The last step of our work was to compare the data obtained from HILIC and RPLC experiments in order to select the optimal combination of extraction conditions and chromatographic strategy.

Data visualized with PCA showed that the different extraction methods clustered in a similar way when analyzed with RPLC and HILIC, suggesting that pH 10 and $CHCl_3$ extraction methods resulted in more pronounced differences in the response of platelet metabolites (Fig. 3).

A total of 151 metabolites were detected in all conditions (Fig. 7). HILIC and RPLC shared about 50% of all metabolites detected. The other half of the detected metabolites was divided in the following way: 46 unique metabolites were detected in HILIC and 31 in RPLC. Thus, the highest number of metabolites was identified during HILIC–MS experiments (121 versus 106) (Table 1). When comparing the results obtained from the different extractions analyzed with two LC–MS approaches, the recoveries of the extraction methods appeared to be independent from the chromatography used. In fact, lower response was always achieved for pH 10 and pH 2

(Fig. 4a). Nevertheless, the chromatography influenced the response of metabolites detected (Fig. 5). For example, less polar amino acids, such as phenylalanine, tryptophan, and (iso)leucine, had a higher response when using RPLC compared to HILIC (Fig. 5a). RPLC did not retain more polar amino acids leading to their co-elution at the same retention time at the beginning of the chromatography and thus increasing their probability to experience ion suppression. Similar behavior was observed for other polar compounds as most of the phosphorylated compounds and of organic acids which were not detected in RPLC or exhibited a lower sensitivity (Fig. 5b–d). On the other hand, fatty acids, eicosanoids, and lipids exhibited an opposite behavior. These compounds were poorly retained in HILIC but resolved well in RPLC. As a consequence of this different chromatographic behavior, we found RPLC to be more suitable for this group of metabolites. In fact, this LC–MS approach was able to separate isomeric and isobaric species as well as to provide a better sensitivity (Fig. 5f). The identified metabolites were divided in different classes, showing that HILIC provides a better coverage with exception of lipids (Fig. 6f).

Comparing the reproducibility achieved by HILIC– and RPLC–MS, our analysis showed that $CHCl_3$ and pH 10 extractions had the lowest RSD% with both chromatographic approaches, while methanol:water in one or two step methods had the best reproducibility (Fig. 6). The lower reproducibility achieved using the LLE protocol could be explained by our choice to combine the organic and aqueous phases after extraction and to process both phases together. An alternative way would have been to dry and run separately the aqueous and organic phase of $CHCl_3$ extracted samples. This would have implied two specific methods for polar and non-polar metabolites, for example, a reversed phase chromatography for organic phase analysis and HILIC for the aqueous phase. We decided to process both phases together in order to find a single optimal protocol.

Based on our results, we can report that HILIC provides a better coverage of the platelet metabolome by detecting 126 metabolites compared to 106 metabolites identified with RPLC. In particular, when combined with pH 7 extraction, HILIC guarantees optimal conditions considering metabolites detected, reproducibility, and time of analysis. This latter criterion is an important factor in metabolomic studies. When comparing the metabolites recovered by the pH 7 and the two-step extraction, we think that the loss of the few metabolites in pH 7 method is well compensated by the fact that this method is much faster, since samples do not need to be lyophilized and reconstituted (see Sections 2.3.1 and 2.3.7).

Some metabolites could be detected both in positive and negative mode. We selected for each metabolite an optimal ionization mode based on their intensity and chromatographic behavior, which has particular importance in HILIC–MS analysis where we used acidic condition in positive mode and basic conditions in negative mode. However, an overlap of identified metabolites was observed between positive and negative ionization in the HILIC– and RPLC–MS analysis (Fig. 4b). Using the pH 7 extraction in HILIC–MS, 38 metabolites were detected both in positive and negative mode, while in RPLC–MS, 23 of the 97 metabolite were shared between negative and positive mode. We combined different chromatographic separations to see if the coverage of the exo-metabolome could be increased. When combining HILIC and RPLC in positive mode an overlap of 50% was obtained (42 on 80 metabolites detected). Better results were obtained when HILIC and RPLC in negative mode were combined, or when HILIC in negative mode was combined with RPLC in positive mode, which led to the identification of 111 metabolites with only 26 metabolites overlapping (Fig. 4b). These findings suggest an alternative protocol in which, after extraction (pH 7), the sample would be analyzed using HILIC–MS in negative mode and RPLC–MS in positive mode.

The obtained results are in agreement with those previously reported by Sellick et al. in which the methanol–water extraction was found to be more effective for CHO cells and whereas acidic and basic conditions resulted in the lowest response [19]. In contrast, there is no agreement with the data by Sana et al. [22] reporting that basic and acidic conditions resulted in a more efficient response than neutral extraction for erythrocytes. In our opinion, these differences are due to the fact that Sana et al. used a liquid–liquid extraction (methanol–chloroform–water), during which acidic and/or basic conditions promote ionization of acidic and/or basic compounds in solution, thus, increasing their solubility in water phase. In our study, the use of LLE did not result in the best metabolites response; subsequently, we did not pursue this approach further.

4. Conclusions

We defined a protocol for metabolite profiling of platelets, in which a methanol:water (7:3) pH 7 extraction was coupled with HILIC–MS method. However, this protocol does not provide a complete recovery of all metabolites, which would require a combination of different extraction methods as well as the use of multiple analytical platforms. We also showed that the number of detected metabolites could be increased when analyzing the samples with two different LC–MS methods or when employing complementary extraction methods in parallel.

We believed that this analytical protocol represents the best compromise between reproducibility, short time analysis, and good coverage, for defining and analyzing the platelet metabolome, and that it is thus suitable for metabolomic studies of platelets storage lesions in transfusion medicine.

Acknowledgement

This work was supported by ERC grant agreement No. 232816 – SYSTEM Us.

References

[1] E. Holmes, I.D. Wilson, J.K. Nicholson, Cell 134 (2008) 714.
[2] E. Holmes, R.L. Loo, J. Stamler, M. Bictash, I.K. Yap, Q. Chan, T. Ebbels, M. De Iorio, I.J. Brown, K.A. Veselkov, M.L. Daviglus, H. Kesteloot, H. Ueshima, L. Zhao, J.K. Nicholson, P. Elliott, Nature 453 (2008) 396.
[3] C. Denkert, J. Budczies, W. Weichert, G. Wohlgemuth, M. Scholz, T. Kind, S. Niesporek, A. Noske, A. Buckendahl, M. Dietel, O. Fiehn, Mol. Cancer 7 (2008) 72.
[4] C. Denkert, J. Budczies, T. Kind, W. Weichert, P. Tablack, J. Sehouli, S. Niesporek, D. Konsgen, M. Dietel, O. Fiehn, Cancer Res. 66 (2006) 10795.
[5] T.A. Clayton, J.C. Lindon, O. Cloarec, H. Antti, C. Charuel, G. Hanton, J.P. Provost, J.L. Le Net, D. Baker, R.J. Walley, J.R. Everett, J.K. Nicholson, Nature 440 (2006) 1073.
[6] W.B. Dunn, Phys. Biol. 5 (2008) 011001.
[7] G. Pendyala, E.J. Want, W. Webb, G. Siuzdak, H.S. Fox, J. Neuroimmune Pharmacol.: Off. J. Soc. NeuroImmune Pharmacol. 2 (2007) 72.
[8] W.B. Dunn, D.I. Broadhurst, H.J. Atherton, R. Goodacre, J.L. Griffin, Chem. Soc. Rev. 40 (2011) 387.
[9] W.B. Dunn, D. Broadhurst, P. Begley, E. Zelena, S. Francis-McIntyre, N. Anderson, M. Brown, J.D. Knowles, A. Halsall, J.N. Haselden, A.W. Nicholls, I.D. Wilson, D.B. Kell, R. Goodacre, Human Serum Metabolome Consortium, Nat. Protoc. 6 (2011) 1060.
[10] E.J. Want, I.D. Wilson, H. Gika, G. Theodoridis, R.S. Plumb, J. Shockcor, E. Holmes, J.K. Nicholson, Nat. Protoc. 5 (2010) 1005.
[11] Z.H. Yu, G. Kastenmuller, Y. He, P. Belcredi, G. Moller, C. Prehn, J. Mendes, S. Wahl, W. Roemisch-Margl, U. Ceglarek, A. Polonikov, N. Dahmen, H. Prokisch, L. Xie, Y.X. Li, H.E. Wichmann, A. Peters, F. Kronenberg, K. Suhre, J. Adamski, T. Illig, R. Wang-Sattler, PLoS ONE 6 (2011).
[12] N. Psychogios, D.D. Hau, J. Peng, A.C. Guo, R. Mandal, S. Bouatra, I. Sinelnikov, R. Krishnamurthy, R. Eisner, B. Gautam, N. Young, J.G. Xia, C. Knox, E. Dong, P. Huang, Z. Hollander, T.L. Pedersen, S.R. Smith, F. Bamforth, R. Greiner, B. McManus, J.W. Newman, T. Goodfriend, D.S. Wishart, PLoS ONE 6 (2011).
[13] E. Werner, J.F. Heilier, C. Ducruix, E. Ezan, C. Junot, J.C. Tabet, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 871 (2008) 143.
[14] E. Nevedomskaya, R. Ramautar, R. Derks, I. Westbroek, G. Zondag, I. van der Pluijm, A.M. Deelder, O.A. Mayboroda, J. Proteome Res. 9 (2010) 4869.

[15] M.M. Koek, F. Bakels, W. Engel, A. van den Maagdenberg, M.D. Ferrari, L. Coulier, T. Hankemeier, Anal. Chem. 82 (2010) 156.
[16] W. Lu, B.D. Bennett, J.D. Rabinowitz, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 871 (2008) 236.
[17] B.D. Bennett, J. Yuan, E.H. Kimball, J.D. Rabinowitz, Nat. Protoc. 3 (2008) 1299.
[18] J.B. Ritter, Y. Genzel, U. Reichl, Anal. Biochem. 373 (2008) 349.
[19] C.A. Sellick, D. Knight, A.S. Croxford, A.R. Maqsood, G.M. Stephens, R. Goodacre, A.J. Dickson, Metabolomics 6 (2010) 427.
[20] S. Dietmair, N.E. Timmins, P.P. Gray, L.K. Nielsen, J.O. Kromer, Anal. Biochem. 404 (2010) 155.
[21] C.A. Sellick, R. Hansen, G.M. Stephens, R. Goodacre, A.J. Dickson, Nat. Protoc. 6 (2011) 1241.
[22] T.R. Sana, K. Waddell, S.M. Fischer, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 871 (2008) 314.
[23] Y. Zhang, A. Jiye, G. Wang, Q. Huang, B. Yan, W. Zha, S. Gu, L. Liu, H. Ren, M. Ren, L. Sheng, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 877 (2009) 1751.
[24] Z.M. Ruggeri, Nat. Med. 8 (2002) 1227.
[25] A.S. Weyrich, S. Lindemann, G.A. Zimmerman, J. Thromb. Haemost. 1 (2003) 1897.
[26] P. Schubert, D.V. Devine, Vox Sang. 99 (2010) 112.
[27] M. Oresic, J. Tang, T. Seppanen-Laakso, I. Mattila, S.E. Saarni, S.I. Saarni, J. Lonnqvist, M. Sysi-Aho, T. Hyotylainen, J. Perala, J. Suvisaari, Genome Med. 3 (2011) 19.
[28] K. Spagou, I.D. Wilson, P. Masson, G. Theodoridis, N. Raikos, M. Coen, E. Holmes, J.C. Lindon, R.S. Plumb, J.K. Nicholson, E.J. Want, Anal. Chem. 83 (2011) 382.
[29] G. Paglia, S. Hrafnsdottir, M. Magnusdottir, R.M. Fleming, S. Thorlacius, B.O. Palsson, I. Thiele, Anal. Bioanal. Chem. 402 (2012) 1183.