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Serum or plasma, what is the difference? Investigations to facilitate the sample material selection decision making process for metabolomics studies and beyond

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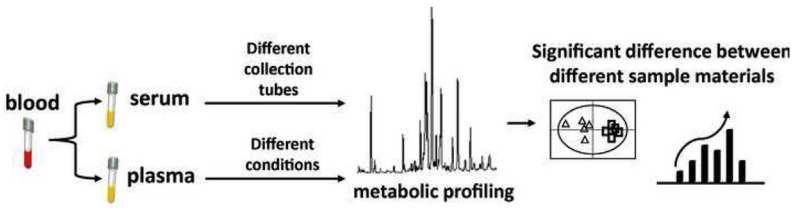
HIGHLIGHTS

- Plasma and serum are commonly used, but generated by different blood processing procedures.
- Coagulation and associated processes distinctly alter serum metabolite levels.
- Metabolite profiles of serum vs plasma showed 46% out of 216 metabolites had significant different levels.
- No differences in Metabolite profiles were detected in the comparison of standard and platelet-free plasma.

GRAPHICAL ABSTRACT



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ABSTRACT

In analytical chemistry serum as well as plasma are recommended as sample material of choice. However, blood processing for the generation of serum or plasma is rather different. Whether plasma or serum is the preferable sample material is still controversial discussed. We performed in paired samples three UHPLC-mass spectrometry-driven metabolomics studies. In study 1 metabolite profiles of serum vs plasma were compared. 46% out of 216 identified metabolites showed significant different levels (paired Wilcoxon signed-rank test, $p < 0.05$, FDR <0.01) with only three metabolites (methionine, C2:0- and C3:0-carnitine) showing lower levels in serum. In study 2 comparison of three different serum blood collection tubes revealed that coagulation and associated processes distinctly alter metabolite levels depending on the tube-specific clotting process. Most pronounced differences were found for the dipeptide phenylalanine-phenylalanine (highest levels in silicate containing serum blood collection tubes). In study 3 possible adverse effects of platelets, which still remain in standard plasma even after correct processing, were investigated. No differences in a pattern of 216 metabolites were detected in the comparison of standard and platelet-free plasma (PFP). Our results give novel insights in fundamental differences between serum and plasma, thereby providing valuable information for analytical chemists

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for decision making to either use serum or plasma before starting complex and time-consuming analytical investigations.

1. Introduction

Serum and plasma samples are investigated every day in numerous laboratories of analytical chemists. Both sample materials are recommended as sample material of choice, e.g. for metabolomics-based biomarker studies [1–12]. However, the type of sample collection tube as well as the processing of these two materials after blood drawing are rather different. For the generation of serum from whole blood, proper clotting by exposing serum blood collection tubes to room temperature (RT) for a defined time period (usually 30–60 min) is mandatory [13–15]. Serum tubes contain either a coagulation enhancer or no additives. Plasma blood collection tubes, in contrast, contain additives to prevent coagulation. Consequently, these sample tubes can either be centrifuged at once after blood drawing to separate plasma from blood cells or they should be immediately cooled to avoid possible adverse effects of RT, e.g. on metabolite biomarker concentrations [16]. Hence, proper handling of plasma sample tubes is much easier than serum tubes. But, standard plasma is not cell free. Plasma prepared by daily clinical routine procedures, i.e. centrifugation at 1500 to 4000 g for 5–10 min at 4° to 15 °C [14,15,17,18], still contains a considerable proportion of the platelet number originally present in blood. Noteworthy, the platelet count in plasma is typically not controlled and recorded. Very recently, Lesche et al. demonstrated that two different routinely applied centrifugation protocols (1500×*g* for 10 min and 3000×*g* for 5 min) lead to differences in the metabolomic pattern in plasma [19]. The results of studies searching for biomarkers in blood may be greatly affected by the choice of sample material. Therefore, it is worthwhile to reveal differences between plasma and serum in detail and to define factors affecting metabolite levels in serum and plasma to enable a decision about the preferable sample material for the intended analytical goals.

Some metabolomics studies based on liquid chromatography–mass spectrometry (LC–MS) [1,20], gas chromatography–mass spectrometry (GC–MS) [4] and NMR [11], have focused on differences between plasma and serum, or the effects of preanalytical variations on plasma and serum metabolite levels [1,3,4]. The results of these studies demonstrated significant differences in metabolite profiles between plasma and serum. The reported concentrations of lyso-phosphatidylcholines (LPC), lyso-phosphatidylethanolamines (LPE), amino acids (arginine, tryptophan, valine, serine and phenylalanine), glucose, etc., were higher in serum than in plasma, while levels of citrate, pyruvate, urate and lyso-phosphatidylinositol were detected to be higher in plasma [1,3,4,20]. Besides small molecules like metabolites, thromboxane B2, peptides and proteins also showed pronounced differences between plasma and serum [20–23]. Consequently, the results of studies searching for biomarkers in blood may be greatly affected by the choice of sample material. In this context analytical chemists are regularly confronted, e.g. in metabolomics biomarker studies, with the need to decide if the use of serum or plasma is more suitable for the intended analytical goals.

In our study, we gave detailed insights in differences between serum and plasma. We demonstrated for the first time differences in the analytical results between serum samples based on the distinct coagulation process of the selected commercial blood collection tube. Additionally many scientists are not aware that platelets remain in plasma after standard centrifugation. Therefore, we addressed in a third study the possible risk of remaining platelets in plasma after the application of standard centrifugation conditions on the analytical results. Our data can support analytical chemists in their careful considerations whether serum or plasma is the preferable sample material for the intended use.

2. Experimental section

2.1. Reagents and chemicals

Methanol and acetonitrile (HPLC grade) were obtained from Merck (Darmstadt, Germany). NH_4HCO_3 and formic acid used as mobile phase additives were purchased from Sigma-Aldrich (St. Louis, MO, USA). Milli-Q water system (Millipore, Billerica, USA) was used to generate ultrapure water. Chemical standards were purchased from J&K chemicals (Shanghai, China), Sigma-Aldrich (St. Louis, MO, USA), and Avanti polar lipids Inc. (Alabaster, AL, USA). Serum blood collection tubes containing silicate and tubes without coagulation enhancers, as well as K^+-ethylene-diamine-tetraacetate (EDTA) plasma collection tubes were provided by Sarstedt (Nümbrecht, Germany). Rapid Serum Tubes (RST) Vacutainer were provided by Becton Dickinson Company (NJ, USA).

2.2. Sample collection

Paired plasma and serum samples were prepared to perform a general comparison by metabolite profiling (study 1; scheme in Fig. 1). K^+-EDTA blood collection tubes were gently mixed, centrifuged at once after blood drawing (4000 g, 4 °C, 10 min) and plasmas were immediately frozen at −80 °C. Blood intended for the preparation of serum was gently mixed and allowed to clot in an upright position for 60 min at room temperature followed by centrifugation (4000 g, 4 °C, 10 min) and subsequently stored at −80 °C.

For study 2 (scheme in Fig. 1), K^+-EDTA blood collection tubes and three different serum blood collection tubes were used. The serum tubes contained either thrombin for very rapid clotting activation (Rapid Serum Tubes (RST)), or silicate to accelerate coagulation, or no coagulation enhancers. K^+-EDTA blood collection tubes were either centrifuged at once to separate plasma from blood cells or whole blood was exposed for 60 min either to room temperature or to 30 °C before plasma separation (30 °C was chosen to simulate blood processing in a situation during summer time without air condition). Serum blood collection tubes containing thrombin were allowed to clot for 5 min and silicate containing blood collection tubes for 30 min at RT according to the instructions of the manufacturers. Clotting time for serum blood collection tubes without coagulation enhancer was 60 min either at RT or at 30 °C according to the instruction of the manufacturer.

In study 3 (scheme in Fig. 1), different centrifugation conditions were applied using K^+-EDTA blood collection tubes: condition A: standard centrifugation (4000 g, 4 °C, 10 min); condition B: condition A followed by a fast centrifugation step (16,000 g, 4 °C, 10 min); condition C: long centrifugation (4000 g, 4 °C, 30 min); condition D: condition C followed by a fast centrifugation step (16,000 g, 4 °C,

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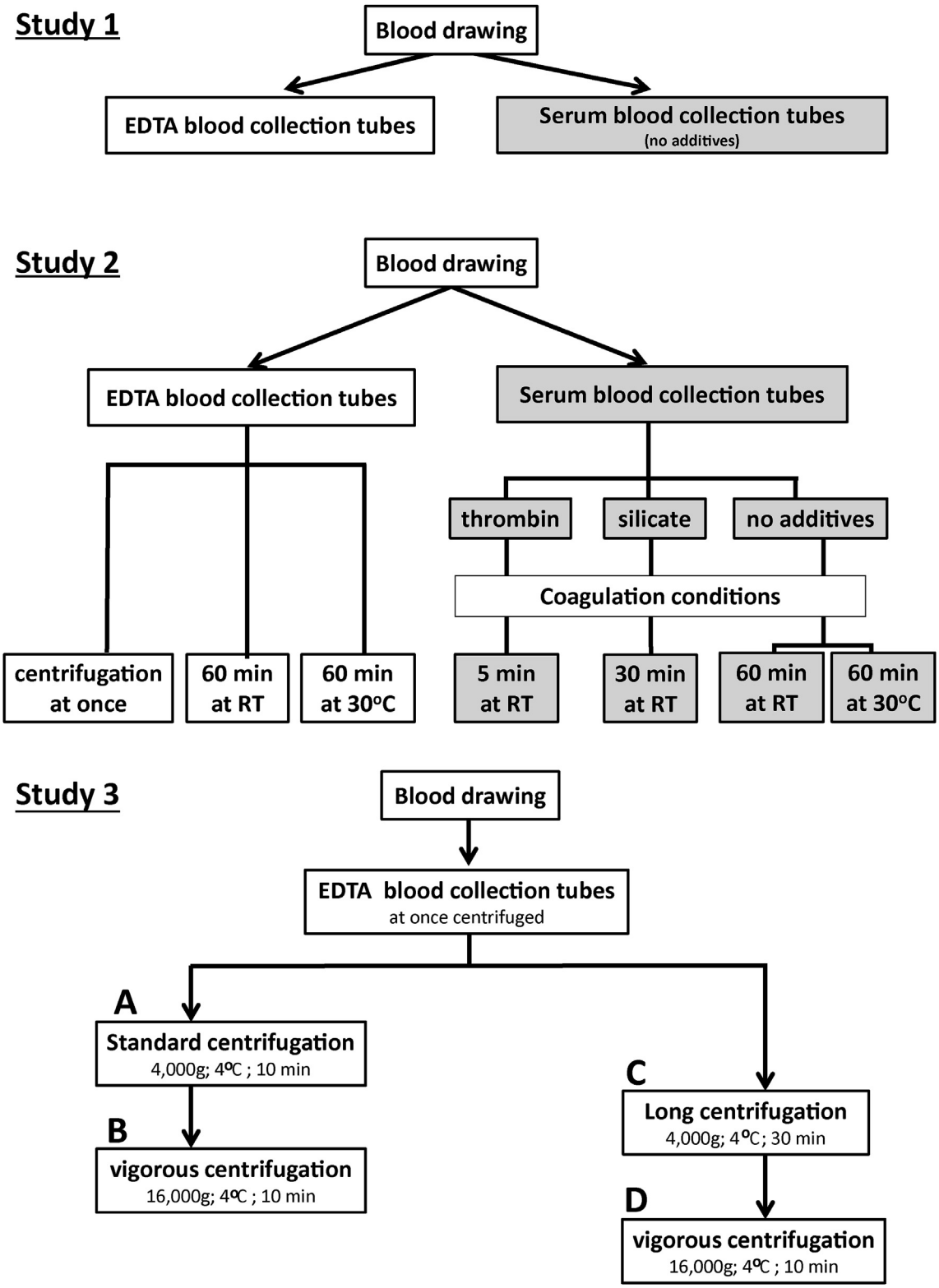

Fig. 1. Scheme of the experimental design of study 1 (n = 10), study 2 (n = 12) and study 3 (n = 10).

10 min). An ADVIA 2120 hematology system (Siemens Health Care, Germany) was used to count the platelets. Blood was drawn from healthy volunteers (study 1: n = 10 (5 male and 5 female); study 2: n = 12 (6 male and 6 female); study 3: n = 10 (5 male and 5 female)). Subsequent to plasma or serum separation all samples were immediately stored in 300 µl aliquots at −80 °C. Informed written consent was obtained and the local medical ethics committee approved the protocol.

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2.3. Sample preparation for metabolite profiling and targeted analyses

The plasma and serum aliquots for study 1 and 3 (metabolite profiling) were thawed in ice water, then 600 µl of acetonitrile containing nine internal standards (carnitine C0-d3, carnitine-C4:0-d3, carnitine C10:0-d3, carnitine C16:0-d3, LPC 19:0, LPC 15:0, PC 34:0, FFA 16:0-d4, FFA C22:0-d4) were added. After 2 min of vortexing, the samples were centrifuged at 13,000 g for 10 min at 4 °C. The supernatant was dried in a vacuum concentrator and redissolved in 300 µl of 20% acetonitrile/water (v/v) for UHPLC-MS analysis. In study 2 a targeted approach for quantitative measurements was applied using acetonitrile containing carnitine C0-d3, carnitine C10:0-d3, LPC 15:0 and phenylalanine-d5 as internal standards. All other the steps were the same as described above.

2.4. Mass spectrometric analysis

In study 1 and study 3 metabolite profiling covering 216 identified metabolites (for details see supplementary Table S1) was performed by using a UHPLC system (LC 30 AD, Shimadzu, Japan) coupled to a TQ 8050 Triple Quadrupole mass spectrometer (Shimadzu, Kyoto, Japan). In the positive electrospray ionization mode, the system was equipped with an ACQUITY™ C_8 BEH column (1.7 µm, 2.1 × 100 mm) and in the negative ionization mode with a T_3 HSS column (1.8 µm, 2.1 × 100 mm; both columns from Waters, Milford, MA, USA). The column temperature was maintained at 50 °C. Mobile phase (A) 0.1% formic acid in water (v/v) and (B) 0.1% formic acid in acetonitrile (v/v) were used in positive ionization mode, while (C) 5 mM NH_4HCO_3 in water and (D) 5 mM NH_4HCO_3 in 95% methanol/water (v/v) were used in negative ionization mode. The gradient for the analysis in the positive mode initially started from 10% B (maintained for 1 min), increased linearly to 40% B in 4 min, and then increased to 100% B in 12 min (maintained for 5 min), and then returned to the initial ratio for 3 min equilibration. In negative mode, the gradient initially started from 100% C (maintained for 1 min), increased linearly to 40% D in 2 min, increased to 100% D in 9 min (maintained for 4 min), and returned to the initial ratio for 4 min equilibration. The mass spectrometric parameters were as follows: heating gas flow (10 l/min); dry gas flow (10 l/min); nebulizing gas flow (3 l/min); DL temperature (250 °C); heat block temperature (400 °C); interface heater temperature (300 °C). In positive and negative modes, the voltages of the capillary (3500 V) and of the nozzle (400 V) were the same (with opposite polarity). Typical chromatograms of a MRM metabolomic analysis from a QC sample performed in the positive and negative ESI modes are given in supplementary Figure S1.

In study 1 additionally 18 amino acids were analyzed by a commercial kit according to the manufacturer's instructions (AccQ Tag™; Waters, Milford, MA, USA) in the positive ionization mode on the same LC-MS system as described above.

2.5. Data analysis

Metabolite identification was accomplished by high resolution mass spectrometer based on exact mass, retention time, and MS/MS pattern, as well as available standard compounds. Lab Solutions (Shimadzu, Kyoto, Japan) was used to extract the peak areas. Each metabolite peak in the profiling approaches was corrected by an internal standard as recently described [24]. SIMCA-P (version 11.0; Umetrics, Umea, Sweden) was applied to perform principal component analysis (PCA) including unit variance (UV) scaling before multivariate analysis. Paired Wilcoxon signed-rank test with a FDR <0.01 was applied. A p value <0.05 was considered statistically significant. The generation of the heatmap was performed with Multiexperiment Viewer (Dana-Farber Cancer Institute, Boston, MA, USA).

3. Results and discussion

Firstly, in study 1 we compared in paired plasma and serum samples a profile of 216 identified metabolites representing a wide spectrum of various (bio)chemical compounds covered by UHPLC-MS (see supplementary Table S1 for a total list of these metabolites). The scores plot from a principal component analysis (PCA) revealed clear differences in the metabolite levels of these profiles (Fig. 2A). Next a heatmap was generated showing the 99 metabolites with significant differences between plasma and serum based on a paired Wilcoxon signed-rank test ($p < 0.05$, FDR <0.01) (Fig. 2B). Supplementary Table S2 shows details of these 99 differential metabolites. Various classes are represented among these 99 metabolites, namely acylcarnitines, free fatty acids (FFA), fatty acid amides (FAA), lyso-phosphatidylcholines (LPC), lyso-phosphatidylethanolamines (LPE), phosphatidylcholines (PC), phosphatidylethanolamines (PE), ether phospholipids (PC-O), and sphingomyelins (SM). Only the levels of three metabolites (carnitine C2:0, carnitine C3:0 and methionine) were significantly lower in serum in our profiling approach (Fig. 2B). No significant differences were detected for most amino acids, bile acids, some medium and long-chain acyl-carnitines, as well as for several phospholipids. A recent metabolomics study, mainly covering phospholipids, reported that 104 of 122 metabolites significantly differed between plasma and serum [1].

How can these differences be explained? Higher LPC and LPE levels in serum [1,25] are likely to be caused by phospholipases released from activated platelets during coagulation [26]. Phospholipases are key enzymes for the generation of LPCs and LPEs. Other reports comparing plasma and serum described higher serum levels of thromboxane B2 [27], as well as characteristic peaks of peptides, hypoxanthine and xanthine [3,12,21,28], and distinct proteins [22,23] in serum. We hypothesize that the detected differences in the metabolite profiles between plasma and serum samples may be caused by metabolites released or modified by activated platelets. Furthermore, enzymes may be activated and/or released during clotting of blood thereby affecting the levels of distinct metabolites. These clotting-associated alterations may be enforced by different coagulation enhancers, clotting times and ambient temperatures (e.g. seasonal differences in a non-air-conditioned room during summertime).

The major goal of study 2 was to compare the potential effect of four common clotting procedures applied in daily clinical practice on metabolite concentrations. Coagulation enhancers, time and temperature effects were studied in paired serum and plasma samples. An overview of the seven different experimental conditions is given in Fig. 1. Blood clotting of the serum samples was initiated by three different standardly applied strategies. These commonly used serum blood collection tubes contained either thrombin, or silicate, or no coagulation enhancer resulting in final clot formation after 5 min, 30 min, or 60 min, respectively. EDTA plasma was either immediately processed after blood drawing or after 60 min exposure of whole blood to room temperature. In addition, processing of whole blood to serum or plasma was performed at 30 °C for 60 min to study possible effects occurring in situations during summer time at locations without air conditioning. Based on the results from study 1 we selected for these investigations the following representative metabolites showing differences between serum and plasma: LPC C18:0 sn-1 as a representative phospholipid; carnitine C2:0 from the class of acylcarnitines; and the dipeptide phenylalanine-phenylalanine (PhePhe).

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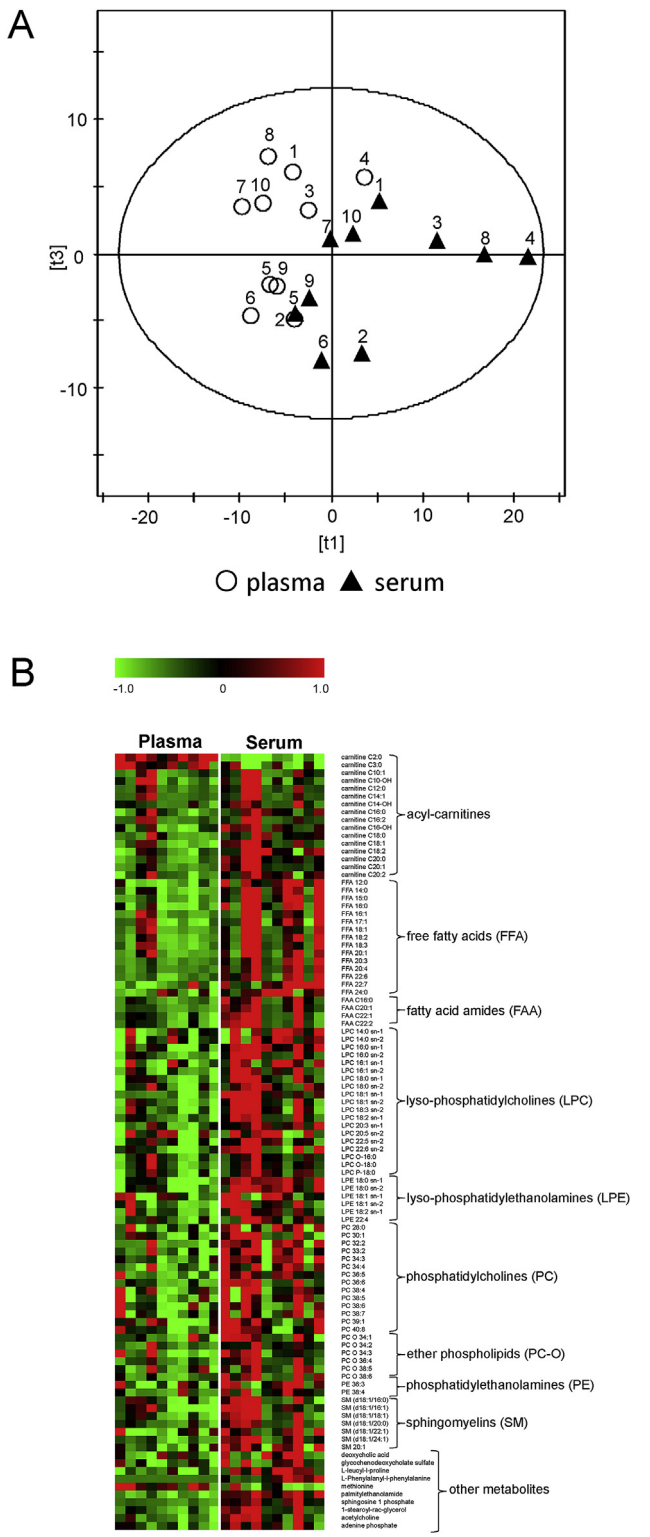

Fig. 2. Comparison of the metabolite profile in paired serum and plasma samples from ten individuals covering 216 metabolites. (A) Principal component analysis (PCA) scores plot of EDTA plasma samples immediately prepared after blood drawing and serum generated in blood collection tubes without coagulation enhancers (clotting: 60 min at room temperature). The numbers indicate the serum and plasma sample pairs originating from the same individual. (B) Heatmap showing 99 out of a total number of 216 metabolites in the pattern with significant differences between plasma and serum based on paired Wilcoxon signed-rank tests ($p < 0.05$, FDR <0.01).

The concentration of the dipeptide PhePhe showed pronounced differences between plasma and serum (Fig. 3). In serum samples, the PhePhe concentrations were markedly influenced by the applied clotting procedure, showing the highest concentrations when silicate was used as coagulation enhancer (Fig. 3). The coagulation enhancement by thrombin alone resulted in lower PhePhe levels than silicate-initiated clotting. Therefore, we hypothesize that this dipeptide is generated during one or several steps of the coagulation cascade of secondary hemostasis. No effect on the PhePhe levels were detected in plasma (Fig. 3). In contrast to PhePhe, the higher serum concentration of LPC C18:0 (Fig. 3), showed a time and temperature dependency. The highest concentrations were detected after 60 min clotting at 30 °C (Fig. 3). This may be a hint for a continuous enzymatic activity of distinct phospholipases released from activated platelets during coagulation in all serum samples [26], i.e. independent from the different serum blood collection tubes used. C2:0-carnitine was one of the three metabolites of the profiling approach showing lower levels in serum (Fig. 2B). The quantification of C2:0-carnitine in the samples of study 2 confirmed the detected difference between plasma and serum of the profiling approach. Study 1 and study 2 revealed that tube type-dependent differences in the coagulation process during serum generation may alter the metabolite pattern in blood. Consequently, usage of different serum collection tubes or different coagulation conditions may lead to inconsistencies in the results of high-resolution profiling approaches. Our data suggest that serum samples originating from different coagulation processes (e.g. thrombin- and silicate-containing sample collection tubes) and/or different clotting times should not be used together in one sample set, at least not for metabolomics biomarker studies. Noteworthy, we achieved also hints that variabilities or differences in clotting temperatures may also affect the results of biomarker studies when serum is used. The consequence of these findings would be to recommend the preferable use of plasma instead of serum.

However, when plasma is chosen as the sample material of choice it should be considered that under daily applied centrifugation conditions of clinical routine laboratories a considerable number of platelets remain in plasma (Table 1). Common centrifugation conditions for blood samples in clinical laboratories reach from 1500 to 4000 g for 5–10 min at 4° to 15 °C [14,15,17,18]. During storage of plasma in a freezer these remaining platelets are lyzed. Up to now it is an open question if these lyzed platelets may cause problems in metabolomics studies investigating plasma.

In study 3 we aimed to analyze the potential effects of these remaining platelets on metabolite profiles. We prepared paired standard and platelet-free plasma (PFP) samples (Fig. 1; study 3). Plasma is entitled as PFP if the platelet number is reduced to <10,000 platelets/μl [29]. PFP can be generated by centrifuging the samples at a higher speed or longer centrifugation time using a standard centrifuge [30].

We applied a standard centrifugation procedure in study 3 (condition A: 4000×g, 10 min, 4 °C) and three different strategies to generate PFP (Fig. 1, conditions B-D). The mean platelet count in whole blood of all subjects was 209,000 platelets/μl (Table 1). Condition A, representing the condition for the preparation of standard plasma, reduced the platelet number to a mean cell count of 27,000 platelets/μl, i.e. 13% of the initial platelet count in whole blood remained (Table 1). All three procedures for the generation of platelet-free plasma (conditions B to D) led to a very similar reduction of the platelet number down to 2000 to 3000 platelets/μl, i.e. down to ca. 1% of the initially platelet count in whole blood (Table 1).

Aiming to figure out potential differences depending on the more than ten-fold higher number of platelets in standard plasma in comparison to PFPs we performed profiling of 216 metabolites

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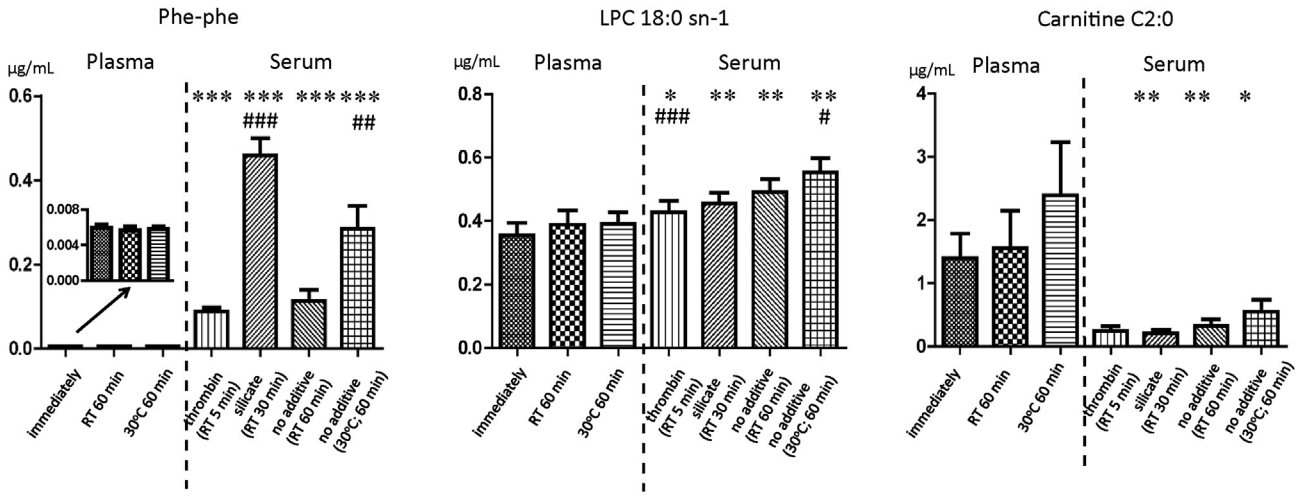

Fig. 3. Targeted analyses of phenylalanine-phenylalanine (PhePhe), LPC C18:0, and acetyl-carnitine in paired EDTA plasma and serum samples collected in three commonly used serum blood collection tubes (paired samples from 12 individuals). In the graphs the clotting time, the additives to enhance coagulation, and the applied temperature are given for the serum samples, respectively. EDTA plasma was either prepared immediately after blood drawing, or the processing time (60 min) and temperature (room temperature or 30 °C) were identical to the condition used for the serum blood collection tubes without coagulation enhancer (no additives). Concentrations are given as mean ± SE; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, vs. plasma that was immediately processed after blood drawing (immediately). Comparisons within serum samples: #: $p < 0.05$, ##: $p < 0.01$, ###: $p < 0.001$, vs. serum without additives clotted for 60 min at room temperature (entitled: "no additive (RT 60 min)".

Table 1
Platelet counts in whole blood and in plasma of 10 healthy volunteers applying different centrifugation conditions given in Fig. 1 and described in the method section.

platelets [1000/µl]												
volunteer	1	2	3	4	5	6	7	8	9	10	mean number	mean % of whole blood
whole blood	295	208	n.d.	n.d.	213	177	227	202	118	228	209	100%
condition A (4000×g, 10 min, 4 °C)	21	20	17	52	38	54	22	17	5	27	27	13%
condition B (cond. A + 16,000×g, 10 min, 4 °C)	2	1	4	1	2	1	5	8	1	6	3	1%
condition C (4000×g, 30 min, 4 °C)	2	1	2	4	5	3	3	2	2	4	3	1%
condition D (cond. C + 16,000×g, 10 min, 4 °C)	1	1	2	2	2	2	2	2	2	3	2	1%

n.d. = not determined.

(supplementary Table 1). These data were evaluated by PCA (Fig. 4). The metabolite fingerprints of all four different plasma preparations from each individual clustered closely together (marked by dashed circles in Fig. 4). A subsequent analysis on the single metabolite level by paired Wilcoxon signed-rank tests ($p < 0.05$; FDR <0.01) revealed that none of the 216 metabolites showed significant differences between routinely prepared standard plasma and platelet-free plasma (data not shown). This finding suggests that in our approach no marked differences between platelet-free and standard plasma occurred.

Based on our data achieved in healthy individuals, we cannot rule out that pathological high platelet numbers may lead to the detection of differences between standard and platelet–free plasma. But it should be considered that the platelet count in extreme pathologically samples is only twice or three times higher than the upper reference range of 450,000 platelets/µl in healthy individuals [31]. Noteworthy, in our study a ten-fold difference in the platelet number between standard and platelet-free plasma samples resulted in no significant difference of the profiled plasma metabolome.

The selected centrifugation force is an important factor affecting the efficiency to reduce the platelet number. Comparing centrifugation at 1500×g for 10 min vs. 3000×g for 5 min, Lesche et al. recently reported significant differences in the platelet number in plasma between the two conditions [32]. More important, also significant differences in the investigated metabolite pattern were detected [32]. The authors conclude that centrifugation conditions

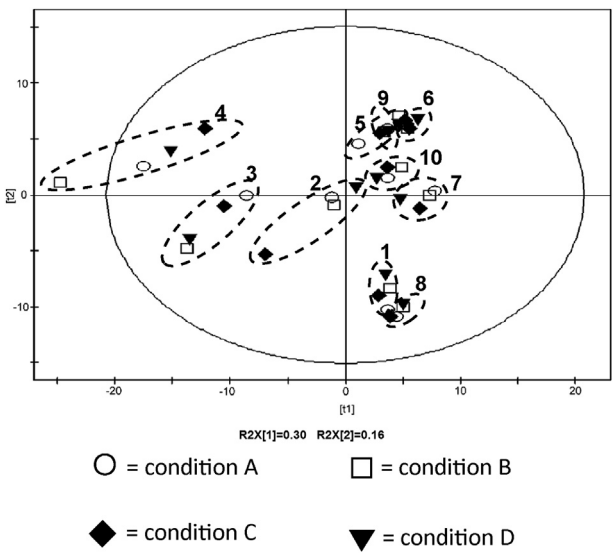

Fig. 4. Comparison of paired standard and platelet-free plasma from ten individuals. PCA scores plot of standard plasma (condition A), and three differently prepared platelet-free plasma samples (conditions B-D). An overview of the experimental design and the applied centrifugation conditions A to D is given in Fig. 1 (study 3). All plasma samples originating from one individual are circled by a dash-dotted line and labeled by a number.

need to be harmonized to ensure comparability of the achieved metabolomics results if plasma is collected at different sites (e.g. in multicenter studies). In our study, centrifugation at 4000×*g* for 10 min, no differences in the metabolite pattern were detected in comparison to platelet-free plasma. Therefore, we recommend this centrifugation condition for the generation of standard plasma, at least for metabolomics-driven biomarker studies.

4. Conclusions

The choice of sample material may greatly affect the results of studies searching for biomarkers in blood, in particular in cases when samples originate from different hospitals in multicenter studies and/or they are collected in the absence of strict standard operating procedures (SOPs).

Based on our results, we suggest that processing of serum samples including sample tube, clotting time and temperature, should be carefully checked before starting the investigation of these samples. It may be risky to combine serum samples generated by different clotting procedures (e.g. initiated by thrombin-vs. silicate-enhanced) into one set of samples. The same holds true for samples exposed to different clotting times. Concerning the use of plasma, we achieved in our metabolite pattern no hint that there is a need to generate platelet-free plasma. However, centrifugation conditions for the generation of plasma should be harmonized if the samples are collected at different places, e.g. in multicenter studies. Based on the different potential sources for variabilities in the composition of serum, we recommend plasma collected and generated strictly according to SOPs as preferable sample material for biomarker studies.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.aca.2018.03.009.

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