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Evaluation of Metabolite Profiles to Determine Short-Term Storage Stability Following Protein Precipitation of Plasma Samples

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MAIN POINTS

- Base peak chromatograms remained broadly similar after one week of storage under the tested conditions.
- Exploratory PCA detected global profile differences that were not evident from visual chromatogram comparison.
- Both storage temperature and whether extracts were stored dry or after reconstitution contributed to multivariate variation.
- Processed plasma extracts should be handled under a uniform storage protocol within the same metabolomics study.

ABSTRACT

Aim: This study aimed to evaluate whether one-week storage temperature and storage state influence the global metabolite profiles of processed plasma extracts following acetonitrile-based protein precipitation.

Materials and Methods: Commercial pooled human plasma was precipitated with acetonitrile, vacuum-dried, and assigned to five conditions: fresh analysis without storage; storage after reconstitution at −20 °C or −80 °C; and storage as dried residues at −20 °C or −80 °C for one week. Six independently prepared vials were analyzed per condition, with duplicate technical injections averaged at the vial level. Metabolite profiles were acquired by Q-TOF LC-MS, processed in MZmine 2.53, normalized by total peak area, and evaluated by principal component analysis (PCA) using MetaboAnalyst.

Results: Representative base peak chromatograms showed broadly similar overall profiles across conditions. In contrast, exploratory PCA revealed storage-condition-associated clustering. For fresh versus solution-stored samples, PC1 and PC2 explained 30.7% and 8.1% of the total variance, respectively. Dry-versus-solution comparisons showed PC1/PC2 contributions of 22.1%/12.2% at −20 °C and 36.1%/10.7% at −80 °C.

Conclusion: Both storage temperature and storage state may contribute to pre-analytical variation in processed plasma extracts. Uniform post-precipitation handling, storage, and injection timing should therefore be maintained within LC-MS-based metabolomics studies.

Keywords: Metabolomics, Plasma, Liquid Chromatography-Mass Spectrometry, Specimen Handling

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INTRODUCTION

Metabolomics is the comprehensive analysis of small molecules that reflect the biochemical state of a biological system. Because metabolites are closely associated with cellular activity, physiological regulation, and pathological alterations, metabolomics has become a powerful approach for biomarker discovery, disease characterization, and mechanistic interpretation in clinical and biomedical research[1–3]. In LC-

MS-based metabolomics, the reliability of the obtained metabolic profile depends not only on instrumental performance but also on the control of pre-analytical variables during sample collection, preparation, storage, and analysis.

The human metabolome is chemically diverse, comprising metabolites with a wide range of molecular weights, polarities, volatilities, acid–base properties, and chemical stabilities[4]. Therefore, maintaining the stability of all metabolites during sample handling is challenging. Several pre-analytical factors, including storage temperature, storage duration, freeze–thaw cycles, enzymatic activity, oxidation, hydrolysis, and solvent composition, may alter metabolite abundances and affect the reproducibility of metabolomics data[5–7]. Such variations are particularly important in plasma metabolomics, where proteins, enzymes, salts, lipids, and low-molecular-weight metabolites coexist in a complex biological matrix.

Protein precipitation with organic solvents such as acetonitrile or methanol is widely used in plasma metabolomics because it provides a simple and efficient way to remove proteins while extracting a broad range of metabolites. However, after protein precipitation, the obtained supernatant or dried residue may need to be stored before LC-MS analysis, especially when large sample sets are prepared, when batch analysis is planned, or when unexpected instrumental delays occur. In such cases, inconsistent storage conditions may introduce additional analytical variability and compromise the comparability of samples within the same study.

Several studies have emphasized that pre-analytical handling can substantially influence blood-based metabolomics data. For instance, Gegner et al. demonstrated that pre-analytical factors such as sample handling, processing time, and temperature contribute to variability and bias in plasma and serum metabolomic and proteomic measurements, supporting the need for standardized pre-analytical protocols[8]. Sample reconstitution conditions are also critical in LC-MS-based metabolomics: Lindahl et al. demonstrated that the composition of the reconstitution solvent can influence metabolome coverage in reversed-phase LC-MS metabolomics, with solvent polarity affecting the detection of compounds across different physicochemical classes[9]. Therefore, the stability of processed plasma extracts after protein precipitation may depend not only on storage temperature and duration, but also on whether the dried residue is stored before or after reconstitution.

Previous studies have evaluated the effects of storage temperature, storage duration, and freeze–thaw cycles on serum or plasma metabolite stability[5–7,10–13]. These studies have demonstrated that many metabolites remain relatively stable under appropriate storage conditions, whereas specific metabolite classes, including amino acids, lipids, biogenic amines, and energy-related metabolites, may be more susceptible to storage-induced changes. However, most available studies have focused on unprocessed plasma/serum samples, long-term storage, or repeated freeze–thaw conditions. Less attention has been given to the short-term stability of processed plasma extracts after protein precipitation, particularly when the extract is stored either as a dried residue or after reconstitution prior to LC-MS injection.

What is already known on this topic?:

- Pre-analytical handling and storage can alter plasma and serum metabolomics profiles.
- Most previous stability studies have focused on unprocessed biospecimens, long-term storage, or freeze–thaw effects rather than short-term storage of processed extracts.

What does this study add on this topic?:

- One-week storage after protein precipitation can produce detectable multivariate differences even when overall chromatographic profiles remain similar.
- Storage state (dry residue versus reconstituted extract) as well as storage temperature can contribute to variability in processed plasma metabolomics samples.

There is currently no universally standardized approach for the short-term storage of processed plasma extracts in LC-MS-based metabolomics. This gap is practically relevant because processed samples are often stored for several days before injection in routine metabolomics workflows. Therefore, this study aimed to evaluate the effect of short-term storage conditions on the metabolite profiles of plasma samples following protein precipitation. Human plasma samples were precipitated with acetonitrile, dried under vacuum centrifugation, and then stored for one week under different conditions, including storage as dried residues or after reconstitution in acetonitrile:water (1:1, v/v) at −20 °C or −80 °C. Freshly prepared samples analyzed without storage were used as the reference condition. The resulting metabolite profiles were analyzed using Q-TOF LC-MS, and global profile differences were evaluated using multivariate analysis. This study was designed to provide practical information on how short-term storage after protein precipitation may influence plasma metabolomics data and to emphasize the importance of consistent sample handling protocols.

MATERIALS AND METHODS

Chemicals and Materials:

LC-MS grade acetonitrile and water were used for chromatographic analysis and sample preparation. Formic acid was used as a mobile phase additive at a final concentration of 0.1% (v/v). Pooled human plasma was used as the biological matrix for the short-term storage stability experiments. All solvents and reagents were allowed to reach the appropriate working conditions before sample preparation, and all prepared samples were handled under identical conditions to minimize pre-analytical variability.

Preparation of the Sample Set:

The study was designed to evaluate the effect of short-term storage conditions on metabolite profiles of plasma extracts after protein precipitation. Pooled human plasma was obtained from a commercial supplier and used as the biological matrix. Six independently prepared LC-MS vials were generated for each experimental condition. For protein precipitation, 100 µL of pooled human plasma was mixed with 200 µL of acetonitrile, corresponding to a plasma-to-acetonitrile ratio of 1:2 (v/v). The samples were vortex-mixed for 1 min and centrifuged at 9000 rpm for 10 min. The resulting supernatants were transferred to clean tubes without disturbing the protein pellet.

The collected supernatants were evaporated to dryness using a Labconco CentriVap Vacuum Concentrator equipped with a methanol trap (Labconco Corporation, Kansas City, MO, USA) at 30 °C under default operating conditions. The obtained dried residues were then divided into different experimental groups to evaluate the influence of storage temperature and storage state on metabolite stability over a one-week period.

The experimental groups were defined as follows:

Fresh group: dried residues were immediately reconstituted in acetonitrile:water (1:1, v/v) and analyzed without storage.

R-20 group: dried residues were immediately reconstituted in acetonitrile:water (1:1, v/v) and then stored at −20 °C for one week before LC-MS analysis.

R-80 group: dried residues were immediately reconstituted in acetonitrile:water (1:1, v/v) and then stored at −80 °C for one week before LC-MS analysis.

D-20 group: dried residues were stored at −20 °C for one week without reconstitution. Reconstitution in acetonitrile:water (1:1, v/v) was performed immediately before LC-MS analysis.

D-80 group: dried residues were stored at −80 °C for one week without reconstitution. Reconstitution in acetonitrile:water (1:1, v/v) was performed immediately before LC-MS analysis.

After reconstitution, all samples were vortex-mixed and transferred to LC-MS vials. Six independently prepared vials were analyzed for each experimental condition using an injection volume of 2 µL. Each vial was injected in duplicate. The two technical injections were averaged at the vial level, yielding six independent observations per experimental condition for multivariate analysis; no averaging was performed across the six independently prepared vials. The injection sequence was randomized to minimize possible run-order effects.

Q-TOF LC-MS Analysis:

Metabolomic analysis was performed using an Agilent 6530 Q-TOF LC-MS system (Agilent Technologies, Santa Clara, CA, USA) operated in positive electrospray ionization mode. Chromatographic separation was achieved on a Zorbax C18 column (2.1 × 100 mm, 1.8 µm; Agilent Technologies, Santa Clara, CA, USA). The mobile phase consisted of water containing 0.1% formic acid as solvent A and acetonitrile containing 0.1% formic acid as solvent B.

The flow rate was maintained at 0.30 mL/min, and the column temperature was set at 35 °C. The gradient elution program started at 10% B and increased linearly to 45% B within the first 5 min. The proportion of solvent B was then increased to 90% by 15 min, returned to 10% B at 18 min, and maintained at the initial conditions until the end of the 25-min run to allow column re-equilibration. The injection volume was 2 µL.

The Q-TOF mass spectrometer was operated in full-scan mode over an m/z range of 100–1700. The drying gas temperature was set at 350 °C, and the capillary voltage was 4000 V. As analytical quality-control measures, each independently prepared vial was injected in duplicate, and the injection sequence was randomized to minimize possible run-order effects. The two technical injections were averaged at the vial level before downstream statistical analysis. No separate pooled QC injection series was included in this short-term storage-comparison experiment.

Data Processing:

Raw LC-MS data files acquired in Agilent .d format were converted to mzML format using ProteoWizard. The converted mzML files were processed using MZmine 2.53 according to the parameters previously optimized and routinely used in our LC-MS-based metabolomics workflow. Briefly, the data processing workflow included mass detection, chromatogram building, chromatographic peak deconvolution, isotope grouping, retention time alignment, gap filling, and peak area extraction.

Mass detection was performed using an intensity threshold of 1.0×10^3. Chromatogram building was performed using a mass tolerance of 10 ppm and a minimum time span of 0.01 min. Chromatographic peak deconvolution was carried out using the local minimum search algorithm. Isotope grouping was performed using a mass tolerance of 10 ppm and a retention time tolerance of 0.1 min. Retention time alignment was performed using a retention time tolerance of 0.2 min and an m/z tolerance of 0.01. Gap filling was applied after alignment to reduce missing values in the final data matrix. The final data matrix consisted of aligned features defined by m/z, retention time, and peak area.

Peak areas from duplicate technical injections of each vial were averaged before statistical analysis. The resulting vial-level peak areas were normalized to the total peak area of each sample to reduce injection-related and technical variability. The normalized feature matrix retained six independent vial-level observations for each experimental condition.

Statistical Analysis:

Principal component analysis (PCA) was performed using the MetaboAnalyst web platform (Xia Lab, McGill University, Montreal, QC, Canada) as an unsupervised exploratory multivariate analysis. Before PCA, the vial-level feature matrix was normalized by total peak area as described above; no additional data transformation or scaling was applied in MetaboAnalyst. Each point in the PCA score plots represents one independently prepared LC-MS vial (n = 6 per group) after averaging its duplicate technical injections. Figure 2 compares the Fresh group with samples stored in solution at −20 °C (R-20) and −80 °C (R-80). Figure 3 compares dry-stored (D-20) and solution-stored (R-20) samples at −20 °C, whereas Figure 4 compares dry-stored (D-80) and solution-stored (R-80) samples at −80 °C. PCA was used to visualize overall storage-condition-associated variation and was not treated as a formal inferential test of group separation.

RESULTS AND DISCUSSION

The present study evaluated the effect of short-term storage conditions on the global metabolite profiles of plasma extracts following protein precipitation. Plasma samples were precipitated with acetonitrile, dried under vacuum centrifugation, and stored for one week either as dried residues or after reconstitution. Freshly prepared samples analyzed without storage were used as the reference condition. This design allowed us to evaluate whether storage temperature and storage state could introduce measurable variation in LC-MS-based metabolomics data.

Representative base peak chromatograms (BPCs) of the plasma extracts are shown in Figure 1. Visual comparison of the BPCs indicated broadly similar chromatographic profiles among the tested conditions. No major chromatographic shift, severe signal loss, or obvious profile distortion was observed between freshly prepared samples and stored samples. This suggests that the tested short-term storage conditions did not markedly affect the gross chromatographic appearance of the plasma extracts. However, visual inspection of BPCs provides only a general overview of the chromatographic profile and may not be sensitive enough to detect subtle or systematic differences in individual metabolite features.

The LC-MS data were processed using MZmine, and the resulting feature matrix was evaluated by PCA. Figure 2 compares freshly analyzed samples with samples stored in solution at −20 °C and −80 °C (n = 6 independent vials per group). The three groups showed storage-condition-associated clustering. PC1 and PC2 accounted for 30.7% and 8.1% of the total variance, respectively. Although the overall BPC profiles appeared similar, this exploratory pattern indicates that one-week storage after reconstitution may introduce systematic variation in the global metabolite profile. The clustering should not be interpreted as formal statistical evidence of group separation.

The clustering pattern in Figure 2 suggests that freshly prepared samples differed from solution-stored samples and that storage temperature contributed to the observed variation. This finding is practically relevant because large metabolomics sample sets cannot always be prepared and injected immediately. If fresh and stored samples are combined within the same analytical study, storage-related variation may become confounded with biological variation. Therefore, all samples within a study should be handled using a uniform storage protocol.

Storage-state-associated variation at −20 °C was evaluated by comparing samples stored as dried residues (D-20) with samples stored after reconstitution (R-20). As shown in Figure 3, the groups displayed distinguishable but overlapping distributions. PC1 and PC2 accounted for 22.1% and 12.2% of the total variance, respectively. The pattern suggests moderate variation associated with the presence or absence of reconstitution solvent during storage. Such variation may reflect differences in solubility, chemical stability, hydrolysis, oxidation, or matrix–solvent interactions.

The corresponding storage-state comparison at −80 °C is shown in Figure 4. Samples stored in solution (R-80) and samples stored as dried residues (D-80) formed different PCA distributions, primarily along PC1, while the dry-stored group showed greater within-group dispersion. PC1 and PC2

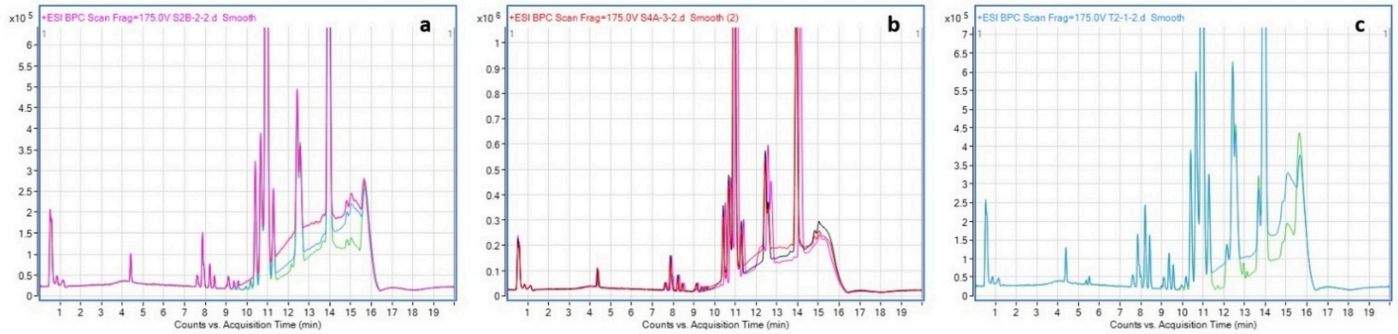


Figure 1. Representative base peak chromatograms (BPCs) of plasma extracts under the tested storage conditions: (a) samples stored at −20 °C for one week, (b) samples stored at −80 °C for one week, and (c) Fresh samples analyzed immediately without storage. The chromatograms are presented for qualitative comparison of the overall chromatographic profiles.

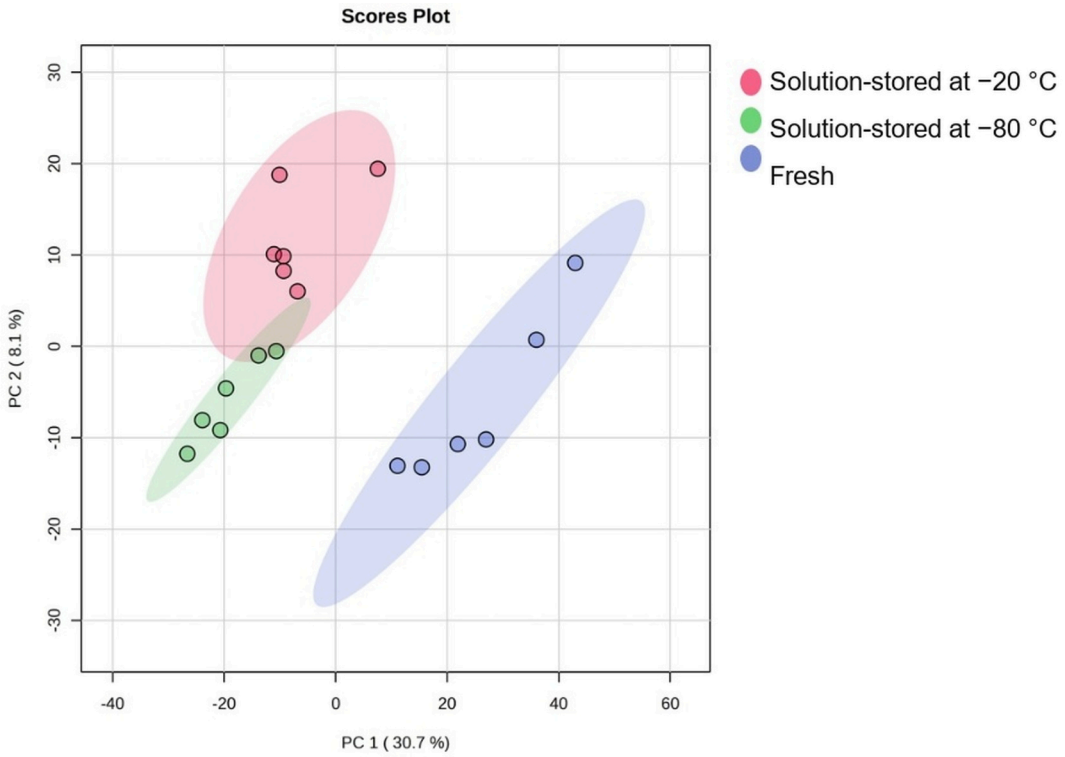


Figure 2. PCA score plot comparing Fresh samples (analyzed without storage), R-20 samples (reconstituted and stored at −20 °C for one week), and R-80 samples (reconstituted and stored at −80 °C for one week). Each point represents one independently prepared vial after averaging duplicate technical injections (n = 6 per group). The shaded ellipses represent the distribution of each experimental group. PC1 and PC2 explain 30.7% and 8.1% of the total variance, respectively.

accounted for 36.1% and 10.7% of the total variance, respectively. This exploratory result indicates that storage state may also contribute to global profile variation at −80 °C, although the PCA does not constitute a formal significance test.

Previous studies have shown that storage conditions can affect metabolite stability in plasma and serum metabolomics. Haid et al. investigated the long-term stability of human plasma metabolites stored at −80 °C and reported that

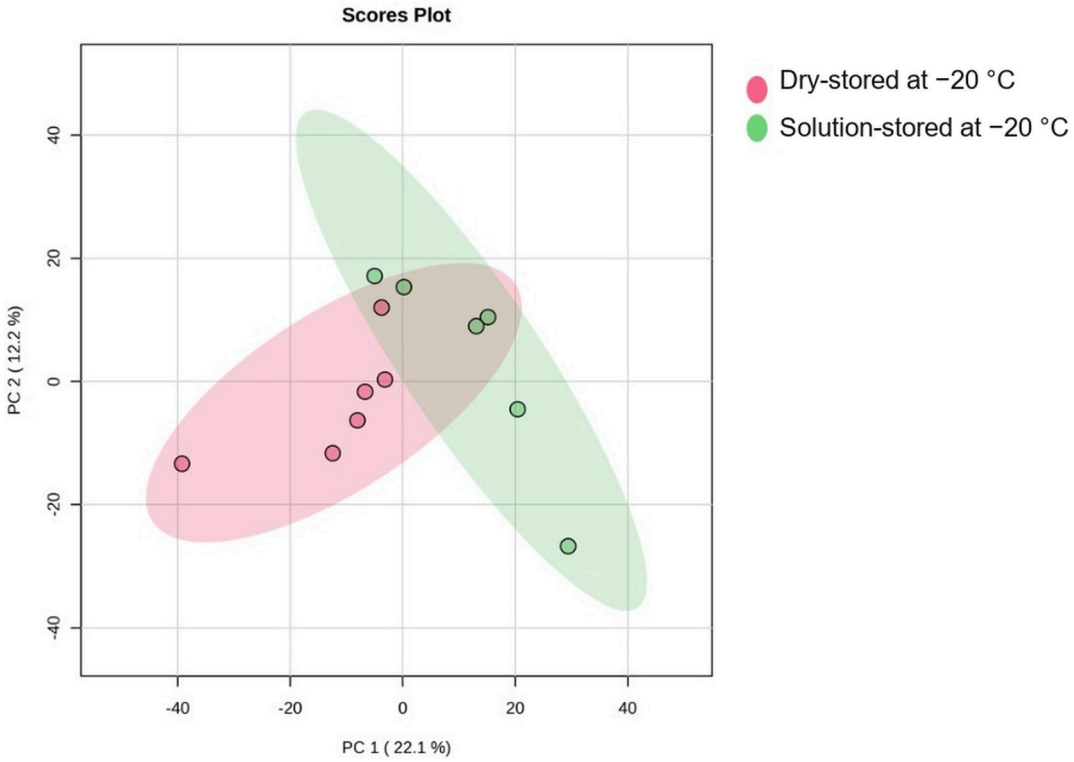


Figure 3. PCA score plot comparing D-20 samples (stored as dried residues at −20 °C for one week and reconstituted immediately before LC-MS analysis) with R-20 samples (reconstituted before storage and stored at −20 °C for one week). Each point represents one independently prepared vial after averaging duplicate technical injections (n = 6 per group). The shaded ellipses represent the distribution of each experimental group. PC1 and PC2 explain 22.1% and 12.2% of the total variance, respectively.

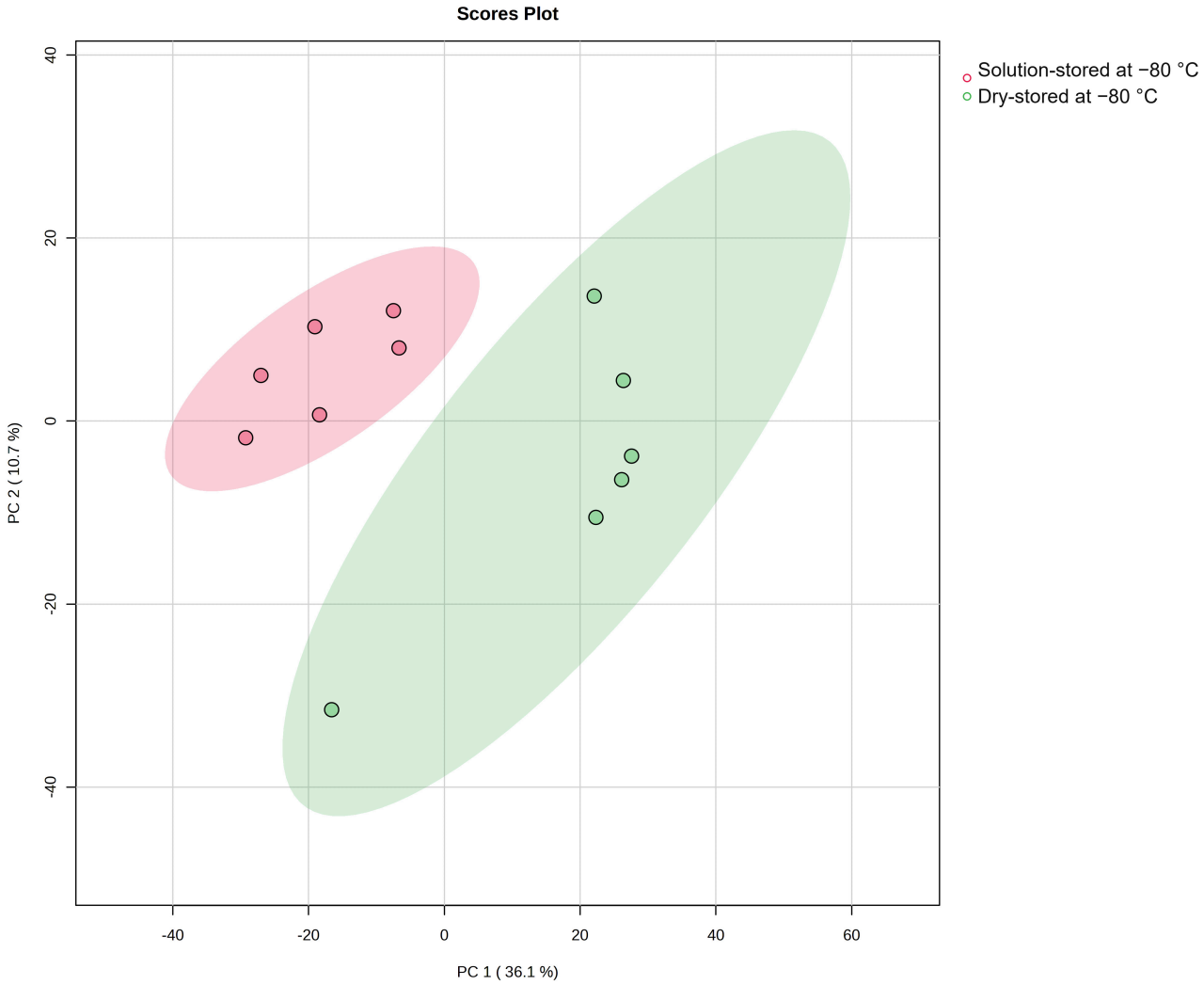


Figure 4. PCA score plot comparing D-80 samples (stored as dried residues at −80 °C for one week and reconstituted immediately before LC-MS analysis) with R-80 samples (reconstituted before storage and stored at −80 °C for one week). Each point represents one independently prepared vial after averaging duplicate technical injections (n = 6 per group). The shaded ellipses represent the distribution of each experimental group. PC1 and PC2 explain 36.1% and 10.7% of the total variance, respectively.

while many metabolites remained stable, several metabolite classes showed concentration changes over time, particularly amino acids, lipids, and hexoses[10]. Wagner-Golbs et al. also evaluated long-term storage of EDTA plasma at −80 °C and showed that most metabolites were stable for several years, whereas longer storage periods affected a larger proportion of metabolites, especially complex lipids, fatty acids, energy metabolism-related molecules, and amino acids[11]. These studies demonstrate that storage at low temperature does not completely eliminate the possibility of metabolite alteration, particularly for chemically labile or biologically reactive compounds.

Moriya et al. further demonstrated that human plasma metabolite profiles are influenced by storage temperature and storage duration in non-targeted metabolomics analysis[12]. Similarly, Breier et al. showed that although many metabolites in serum and plasma are relatively stable under appropriate pre-analytical conditions, certain amino acids and biogenic amines are more susceptible to variation[13]. These findings are consistent with the present study in showing that metabolite profiles can be affected by pre-analytical handling, even when samples are stored under commonly accepted low-temperature conditions.

The present study differs from previous reports in its specific focus on processed plasma extracts after protein precipitation. Most earlier studies evaluated unprocessed plasma or serum, long-term storage, or freeze–thaw conditions. In contrast, our study addressed a common practical situation in LC-MS-based metabolomics: the need to store supernatants or dried residues after protein precipitation before LC-MS injection. This step is particularly relevant when large batches are prepared, when instrument availability is limited, or when all samples must be injected in the same analytical sequence to minimize batch effects.

Our findings indicate that the tested storage conditions did not cause major changes in the overall chromatographic profile but produced detectable differences in exploratory PCA patterns. The main implication is not that one condition universally preserves all metabolites better than the others, but that storage conditions should be applied consistently across

samples. Combining freshly analyzed and stored samples, or combining dry-stored and solution-stored samples at either −20 °C or −80 °C, may introduce avoidable pre-analytical variability.

This study has several limitations. It evaluated one acetonitrile-based preparation workflow and one reconstitution solvent, and the results should not be generalized to other protocols without validation. The analysis focused on global LC-MS profiles and exploratory PCA patterns rather than targeted quantification of individual metabolites. In addition, the group sizes were limited to six independent vials per condition; the PCA plots therefore describe multivariate patterns but do not establish statistical separation. Future studies incorporating longer storage durations, freeze–thaw cycles, alternative solvent systems, and metabolite-specific stability measurements would provide a more comprehensive assessment.

Overall, the results emphasize the importance of standardized short-term storage protocols after protein precipitation. Even when BPC profiles appear broadly similar, multivariate analysis may reveal storage-condition-associated differences. Therefore, metabolomics studies should use consistent sample handling, storage state, storage temperature, and injection timing to minimize pre-analytical variability and improve reproducibility.

CONCLUSION

This study evaluated one-week storage of processed plasma extracts after protein precipitation and vacuum drying. The tested storage conditions did not produce marked differences in overall chromatographic appearance. However, exploratory PCA revealed storage-condition-associated differences in global metabolite profiles. The temperature comparison included freshly analyzed samples and samples stored in solution at −20 °C and −80 °C, while parallel storage-state comparisons evaluated dry-stored and solution-stored samples at both temperatures.

The findings indicate that both storage temperature and storage state may contribute to pre-analytical variation. Combining freshly analyzed samples with stored samples, or combining dry-stored samples with solution-stored samples, may introduce avoidable variability. The same handling and storage protocol should therefore be applied consistently to all samples within an LC-MS-based metabolomics study.

Therefore, when immediate LC-MS analysis is not possible, processed plasma extracts should be stored under standardized and uniform conditions throughout the study. The conclusions of this work are limited to the tested acetonitrile-based protein precipitation and acetonitrile reconstitution workflow. Further studies evaluating different precipitation solvents, solvent ratios, reconstitution media, longer storage periods, and metabolite-specific stability profiles would strengthen the understanding of post-precipitation storage effects in plasma metabolomics.

Ethics Commitee Approval: Commercially available pooled human plasma was used solely as an analytical matrix. No human participants were recruited or contacted by the investigators, no biological specimens were collected specifically for this study, and no identifiable or coded personal or clinical information was accessed. Therefore, institutional ethics committee approval and individual informed consent were not required for this study.

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Author Contributions (CRediT):

Ozan Kaplan: Conceptualization; Methodology; Supervision; Funding acquisition; Resources; Investigation; Data curation; Formal analysis; Writing – original draft; Writing – review & editing.

Emine Koç: Resources; Investigation; Data curation; Formal analysis; Writing – original draft.

Duygu Eneş: Resources; Investigation; Data curation; Formal analysis.

Büşra Uçar Eke: Resources; Investigation; Data curation; Formal analysis.

İncilay Süslü: Conceptualization; Methodology; Supervision.

Sacide Altınöz: Conceptualization; Methodology; Supervision.

Mustafa Çelebier: Conceptualization; Methodology; Supervision; Writing – original draft; Writing – review & editing.

Artificial Intelligence Disclosure: The authors declare that ChatGPT (OpenAI, GPT-5.6 Sol) was used during manuscript preparation for language editing, manuscript organization and formatting, and to improve clarity and readability. The AI tool was not used to generate or modify experimental data, perform statistical or metabolomics data analysis, interpret the study results, or formulate the scientific conclusions. All AI-assisted text was critically reviewed and approved by the authors, who take full responsibility for the accuracy, integrity, and originality of the manuscript.

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