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Standardized Pre-analytical and Proteomic evaluation of Serum Proteins in Gastric Carcinoma

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Abstract

Serum represents a highly complex and dynamic proteomic matrix comprising a broad spectrum of circulating proteins with diverse structural and functional properties. These serum proteins, commonly referred to as plasma proteins (excluding clotting factors in serum), play critical roles in molecular transport, immune surveillance, enzymatic regulation, coagulation cascades, and maintenance of homeostasis. Functionally, they participate in the transport of lipids, hormones, vitamins, trace elements, and metabolites, while also contributing to complement activation, protease inhibition, and inflammatory signaling pathways.

Despite the high total protein concentration in serum, the proteome exhibits an extreme dynamic range spanning over ten orders of magnitude. Approximately 22 high-abundance proteins account for nearly 99% of the total serum protein mass, with albumin, immunoglobulins, transferrin, haptoglobin, and fibrinogen being predominant. In contrast, low-abundance proteins, constituting roughly 1% of the serum proteome, include cytokines, growth factors, tissue leakage proteins, and tumor-derived secretory proteins, many of which serve as potential biomarkers. The majority of circulating proteins are synthesized primarily by hepatocytes and intestinal epithelial cells, whereas immunoglobulins (gamma globulins) are produced by B lymphocytes and plasma cells.

In the present study, serum samples collected from patients diagnosed with gastric carcinoma were comparatively analyzed against age-matched healthy controls. Quantitative protein estimation was performed using the Bradford dye-binding assay, and differential protein expression patterns were evaluated through sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Particular emphasis was placed on standardized pre-analytical variables, including sample collection, processing, storage conditions, and freeze–thaw cycles, to minimize proteolytic degradation and analytical variability. The study underscores the critical importance of rigorous serum handling protocols in proteomic investigations aimed at biomarker discovery and disease-associated protein profiling.

Keywords: Serum, Protein Estimation, SDS PAGE, Gastric Cancer

Introduction

Human serum constitutes a complex circulatory biofluid that functions as a carrier of endogenous and exogenous biomolecules throughout the bloodstream. It facilitates the systemic transport of fatty acids,

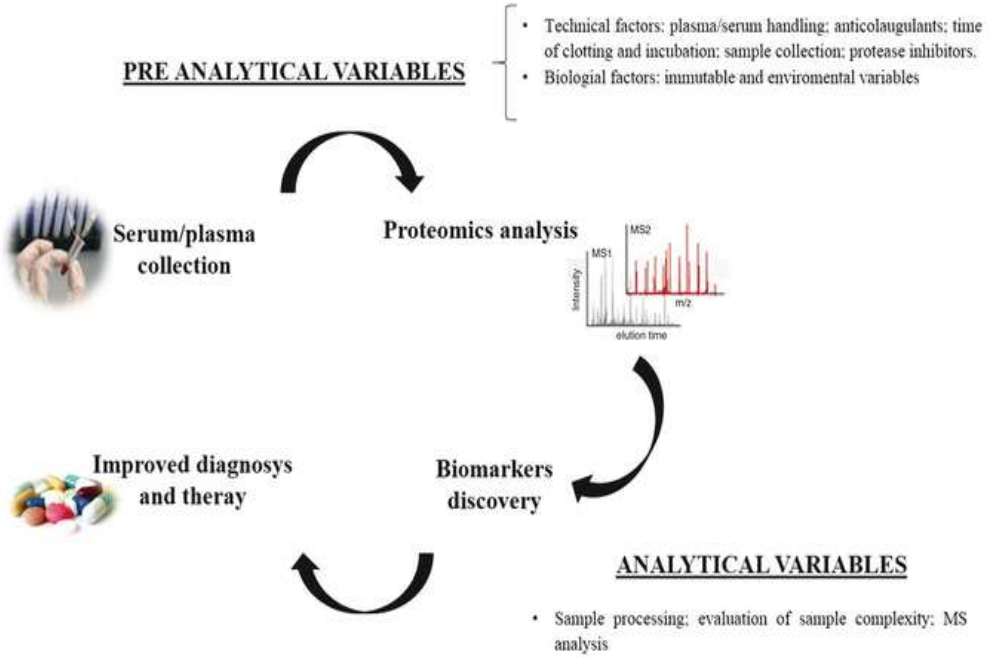
thyroid hormones, metabolites, and signaling molecules that exert regulatory effects on diverse cellular populations across tissues. Owing to its accessibility, relative stability, and rich molecular composition, serum—along with plasma—represents a valuable substrate for large-scale proteomic investigations, particularly in biomarker discovery and in elucidating molecular mechanisms underlying human pathologies (Geyer *et al.,* 2016; Pieragostino *et al.*, 2010).

Intercellular communication within the human body is largely mediated through blood circulation. Consequently, serum and plasma are considered comprehensive reservoirs of physiological and pathological information, reflecting the functional status of multiple organs and tissues. Within the context of the human proteome, the blood proteome is recognized as one of the most complex and dynamic systems. Its protein composition mirrors both homeostatic processes and disease-associated alterations, thereby providing a systemic overview of an individual's health status.

One of the principal advantages of blood-based proteomic analysis is the minimally invasive and standardized nature of sample collection. Although alternative biofluids such as urine and saliva are easier to obtain, they generally exhibit greater variability, reduced stability, and limited systemic representation compared to serum and plasma.

Blood is composed of diverse biochemical constituents including lipids, electrolytes, amino acids, carbohydrates, metabolites, and an extensive repertoire of proteins. It integrates proteins secreted or shed from virtually all tissues and organs, encompassing more than 10,000 distinct protein classes (Thadikkaran *et al.,* 2005; Adkins *et al.,* 2002). However, this estimate only partially represents the true proteomic complexity, which is further amplified by extensive post-translational modifications, alternative splicing events, proteolytic processing, and the vast heterogeneity of immunoglobulin repertoires (Liotta *et al.,* 2003). Notably, approximately 90% of the total serum protein mass is composed of high-abundance proteins (HAPs), such as albumin and immunoglobulins, which create significant analytical challenges in the detection and quantification of low-abundance, disease-associated proteins.

Figure 1: Schematic Representation of Biomarker Discovery



Albumin alone accounts for approximately 50% of the total circulating protein mass, while other high-abundance proteins such as fibrinogen and haptoglobin collectively contribute to nearly 40% of the plasma proteome (Lista *et al.*, 2013). The predominance of these high-abundance proteins (HAPs) significantly increases the analytical complexity of serum and plasma proteomics, primarily due to their overwhelming concentration and extensive binding capacity. Furthermore, the serum proteome complexity is amplified by a broad spectrum of post-translational modifications (PTMs), including glycosylation, phosphorylation, oxidation, and proteolytic cleavage, which generate multiple proteoforms from a single gene product (Anderson and Anderson, 2002).

Although HAPs are often excluded from biomarker discovery pipelines because they mask low-abundance proteins and are considered less specific indicators of disease (Hu *et al.*, 2006), alterations in their concentration, structural variants, or modified forms may serve as sensitive indicators of physiological and pathological changes (Hortin and Sviridov, 2010). Variations in albumin isoforms, acute-phase reactants, and proteolytic fragments can provide clinically relevant insights into inflammatory, metabolic, and neoplastic processes.

In the present study, total serum protein concentration was quantified using the Bradford dye-binding assay, followed by comparative protein profiling through sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). This approach enabled the assessment of overall protein expression patterns and molecular weight distribution profiles in serum samples.

Materials and Methods

Study population and Sample collection All the study subjects included in this study were of South Indian Tamil origin and the ethical approval was granted by the Institutional Ethical committee of Madras Medical College (MMC), Chennai (EC Reg No. ECR/270/Inst./TN/2013; IEC No. 31082015). Patients with primary GC (n=6) admitted in the Dept of Medical gastroenterology, MMC Chennai, were provided with written informed consent and their blood samples were collected from them during the period December 2018 to October 2019. A self-proclaimed healthy individual (n=6) who are free from gastrointestinal diseases and other cancers were recruited as control for the study. Serum from the whole blood sample was separated by C°centrifuging at 3500 rpm for 8 min. Later, the supernatant was collected and stored at -80 until further processing.

Results and Discussion

Serum sample preparation & SDS page analysis

Table 1 describes the sample details. Table 2 describes the demographics of the subjects used in the study.

Table 1: Clinical Characteristics of Patient samples

Sample ID	**Age/Sex**	Malignant Status
1	64/ M	Healthy
2	59/ M	Healthy
3	56/ M	Healthy
4	60/ M	Healthy
5	58/ M	Healthy
6	61/ M	Healthy

7	63/ M	Gastric Cancer
8	57/ M	Gastric Cancer
9	60/ M	Gastric Cancer
10	60/ M	Gastric Cancer
11	63/ M	Gastric Cancer
12	63/ M	Gastric Cancer

Healthy cohorts of South Indian Tamil ethnicity (n=6) who were willing to participate in the study and devoid of gastrointestinal complications, diabetes mellitus, autoimmune diseases were included. Gastric cancer patients (n=6) were enrolled for the study.

Table 2: Demographic of the Subjects used for study

		Healthy control(n=6)	**Gastric cancer(n=5)**
Age(yrs)	Mean + S.D	60+2.7	61+2.4
Gender	**Male**	**6**	**5**

Protein Estimation

Total protein concentration of serum samples was determined using the Bradford dye-binding assay. A standard calibration curve was constructed using Bovine Serum Albumin (BSA) as the reference protein. A working stock solution of BSA (1 mg/mL) was prepared, from which serial dilutions were made to obtain known standard concentrations of 1 µg, 2 µg, 4 µg, 8 µg, 10 µg, and 12 µg. A total of seven standards were prepared to ensure accurate calibration within the selected linear range.

For each standard concentration, the appropriate volume of BSA working solution was transferred into labeled microcentrifuge tubes, and the final volume was adjusted to 1000 µL with Milli-Q water, as detailed in Table 3. Following standard preparation, Bradford reagent was added according to the manufacturer's protocol, and the samples were incubated at room temperature for color development. Absorbance was measured spectrophotometrically at 595 nm. A standard curve was generated by plotting absorbance against protein concentration, and the linear regression equation obtained was subsequently used to calculate the total protein concentration of the serum samples.

Table 3: Details for preparing the standards

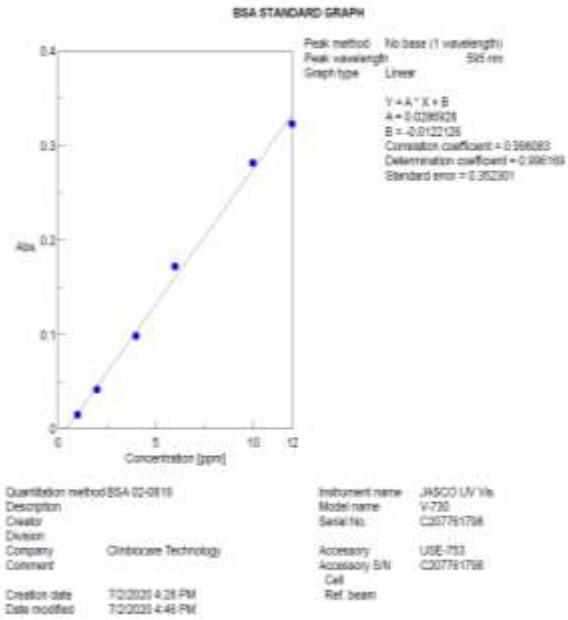
Std Number	**Volume of BSA working concentration (1 mg/ml)**	**Volume of Milli Q water**	**Known concentration of BSA in ug**	**Absorbance OD at 595 nm**
STD 1	1 ul	999 ul	1 ug	0.015
STD 2	2 ul	998 ul	2 ug	0.0416
STD 3	4 ul	996 ul	4 ug	0.0984
STD 4	6 ul	994 ul	6 ug	0.1719
STD 5	8 ul	992 ul	8 ug	0.2577
STD 6	10 ul	990 ul	10 ug	0.2813
STD 7	12 ul	988 ul	12 ug	0.3227

BLANK	-	1000 ul	-	0.000

Following preparation of the BSA standards, 1000 µL of Bradford reagent (Sigma-Aldrich, Germany) was added to each standard tube and mixed thoroughly. The reaction mixtures were incubated at room temperature in the dark for 10 minutes to allow complete color development.

After incubation, absorbance measurements were recorded at 595 nm using a Jasco UV–Visible Dual Beam Spectrophotometer (Model V-730, Maryland, USA). Data acquisition and analysis were performed using Spectra Manager™ II software. A standard calibration curve was constructed by plotting absorbance against known BSA concentrations, and the resulting linear regression equation was used for the quantification of total protein in serum samples. The generated standard curve is presented in Figure 2.

Figure 2: Standard graph with *BSA* for protein estimation



By applying this standard graph, the unknown serum samples were estimated and the concentration of the protein was detailed in table 4.

Table 4: Protein estimation for the serum samples

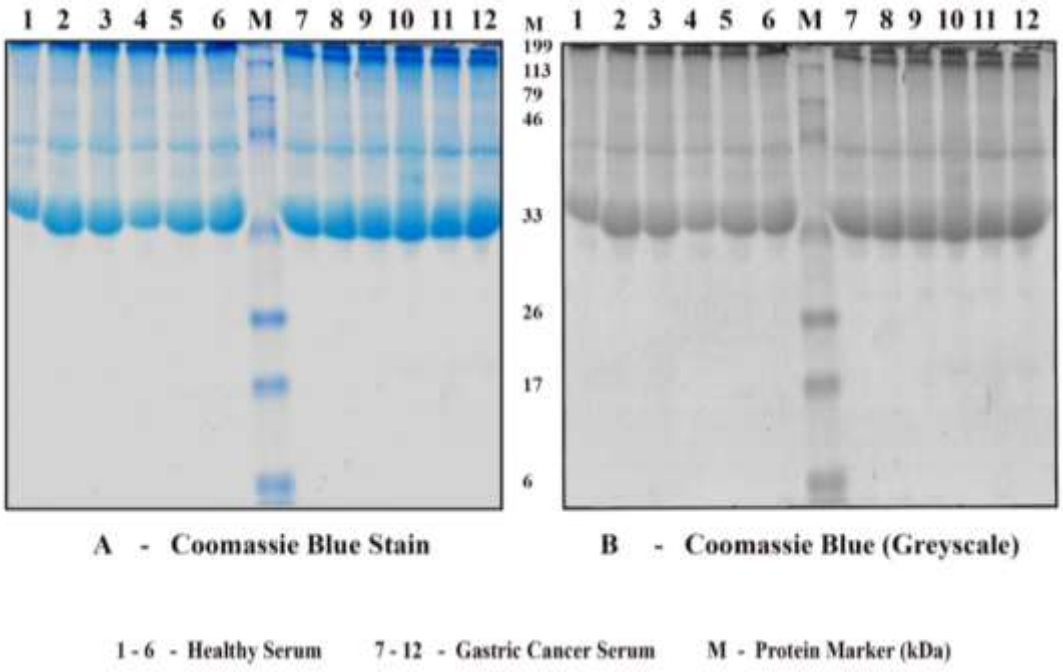
Sample ID	Absorbance OD at 595 nm	Concentration of Protein in ug/ul
1	1.52	52.75
2	1.53	52.94
3	1.50	51.88
4	1.43	49.48
5	1.52	52.52
6	1.65	57.00
7	1.54	53.11
8	1.62	55.87

9	1.59	54.83
10	1.49	51.39
11	1.56	53.80
12	1.63	56.21

SDS-PAGE Analysis

The quantified serum proteins were subsequently subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) to evaluate protein integrity, molecular weight distribution, and comparative expression profiles between study groups. This electrophoretic analysis enabled qualitative assessment of serum protein patterns and detection of variations in band intensity corresponding to differential protein abundance. As illustrated in Figure 3, lanes 1–6 represent serum samples obtained from healthy control subjects, whereas lanes 7–12 correspond to samples collected from patients diagnosed with gastric cancer. A pre-stained molecular weight protein marker was loaded to facilitate estimation of the molecular mass of resolved protein bands and to enable comparative analysis of expression patterns across samples. Following electrophoretic separation, the gels were stained with Coomassie Brilliant Blue R-250 (CBB R-250) to visualize protein bands. Destaining was performed to enhance band clarity and contrast. Comparative analysis of banding patterns was conducted to identify differences in protein expression profiles between healthy and gastric cancer serum samples.

Figure 3 SDS PAGE analysis for serum samples



Sample Selection and Collection

Serum and plasma are frequently considered interchangeable; however, they differ significantly in composition and analytical implications. From a clinical and proteomic standpoint, serum is obtained after coagulation and therefore lacks fibrinogen and certain clotting factors, whereas plasma retains these

components due to anticoagulant treatment. Beyond this distinction, serum and plasma exhibit measurable differences in protein and peptide composition, particularly in low-molecular-weight fractions and coagulation-related proteins (Tammen *et al.,* 2005; Banks *et al.,* 2005). These differences can substantially influence downstream proteomic profiling and biomarker discovery studies.

Mode of Sample Withdrawal

Pre-analytical variables associated with sample collection represent critical determinants of proteomic data reliability. Factors related to both patient condition and laboratory practice can significantly alter protein stability and abundance (Lippi *et al.,* 2005; 2006). Patient posture (supine, sitting, or standing), duration of tourniquet application, venipuncture site, and skin disinfection procedures may influence hemoconcentration, hemolysis, and protein release from blood cells, thereby affecting proteomic outcomes.

To minimize biological variability and potential confounding effects, blood samples were collected in the early morning hours (prior to 10:00 a.m.) under fasting conditions, thereby reducing circadian and metabolic influences on circulating protein levels (Rodriguez and Gonzalez, 2009). A 21-gauge needle was used for venipuncture to reduce the risk of hemolysis and mechanical shear stress. The median cubital vein was selected as the preferred collection site due to its accessibility and reduced likelihood of vascular injury. Prior to venipuncture, the skin was disinfected with alcohol and allowed to air-dry completely to prevent hemolysis or contamination during sample withdrawal.

Careful adherence to standardized collection protocols is essential to preserve proteome integrity and ensure reproducibility in serum-based proteomic investigations.

Anticoagulants

The selection of anticoagulants represents a critical pre-analytical variable in plasma-based proteomic investigations. While serum is obtained after spontaneous coagulation, plasma requires the addition of anticoagulants to prevent clot formation during sample processing. Plasma can be converted to serum at room temperature through thrombin-mediated proteolytic cleavage of fibrinogen and other components of the coagulation cascade (Lista *et al.,* 2013; Capila and Linhardt, 2002; Dammann *et al.,* 2006). Therefore, the choice between serum and plasma—and, in the case of plasma, the selection of an appropriate anticoagulant—constitutes a crucial step in biomarker-oriented studies.

Commonly used anticoagulants include ethylenediaminetetraacetic acid (EDTA), heparin, and citrate. Their use is essential to prevent clotting and preserve plasma integrity; however, each anticoagulant exerts distinct biochemical effects that may influence proteomic outcomes (Lista *et al.,* 2013). The Human Proteome Organization (HUPO) has emphasized the necessity for standardized guidelines concerning anticoagulant usage to ensure reproducibility and comparability across proteomic studies (Rai *et al.,* 2005).

For quantitative proteomics applications, EDTA is frequently recommended due to its ability to chelate divalent metal ions and inhibit metalloprotease activity, thereby reducing proteolytic degradation (Ahn and Simpson, 2007). Nevertheless, EDTA may interfere with assays that depend on divalent cations (e.g., Ca^{2+}- or Mg^{2+}-dependent enzymatic reactions) and has been reported to induce platelet activation or aggregation, potentially altering the plasma proteome composition (White, 2000). Additionally, EDTA may exhibit comparatively lower stability than heparin under certain conditions (Banks *et al.,* 2005).

Heparinized plasma samples are often considered relatively stable (Dammann *et al.*, 2006); however, heparin is a highly negatively charged glycosaminoglycan that can bind not only antithrombin III but also a broad range of plasma proteins (Capila and Linhardt, 2002). This interaction may influence protein–protein binding equilibria and interfere with downstream analytical techniques, particularly chromatographic and mass spectrometric analyses (Lista *et al.*, 2013).

Citrate functions by chelating calcium ions, thereby inhibiting coagulation. However, improper anticoagulant-to-blood ratios may lead to sample dilution and altered protein concentrations, necessitating careful standardization during sample preparation (Rai *et al.*, 2005). Overall, the choice of anticoagulant must be carefully aligned with the experimental objectives, as anticoagulant-specific biochemical interactions can significantly influence plasma proteome composition, protein stability, and analytical reproducibility in proteomic workflows.

Protease Inhibitors

Proteolytic degradation represents a major pre-analytical challenge in serum and plasma proteomics. To preserve protein integrity and prevent post-collection proteolysis, the addition of protease inhibitor cocktails during or immediately after sample collection is strongly recommended. The Human Proteome Organization Human Proteome Project (HUPO-HPP) has emphasized the importance of incorporating protease inhibitors at the earliest stages of sample handling to minimize artificial peptide generation and degradation artifacts (Rai *et al.*, 2005).

Proteomic analyses using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) have demonstrated that plasma samples treated with protease inhibitors exhibit improved stability and reproducibility of peptide profiles compared to untreated samples (Rai *et al.*, 2005). In the absence of inhibitors, ex vivo proteolysis can significantly alter peptide peak intensities and generate misleading biomarker signatures.

Comparative studies have reported that serum and citrate plasma display relatively higher proteolytic activity, followed by heparin plasma and EDTA plasma, although variability depends on handling conditions (Lista *et al.*, 2013). Anticoagulants and additives may influence protease activity either directly or indirectly. Certain compounds can interact with protease active sites, including serine proteases such as trypsin and chymotrypsin, thereby modulating enzymatic activity. Additionally, chemical modifications induced during sample processing may affect amino acid residues, potentially altering protein charge states and shifting isoelectric points (pI). Such alterations can be detected as band smearing or spot distortion in two-dimensional gel electrophoresis (2-DE), reflecting changes in protein stability and post-translational modification patterns. Therefore, strict control of proteolytic activity through the appropriate selection of anticoagulants, rapid sample processing, temperature control, and the use of validated protease inhibitor cocktails is essential to maintain proteome integrity and ensure reproducibility in serum- and plasma-based proteomic investigations.

Collection Tubes

Pre-analytical variables associated with blood collection tubes can significantly influence the integrity and composition of the serum and plasma proteome. Materials used in tube manufacturing—such as silicones, surfactants, polymeric coatings, plasticizers, and separator gels—may leach into the sample and introduce exogenous contaminants. These substances can interfere with downstream proteomic analyses, particularly mass spectrometry-based techniques, by generating background signals or altering

ionization efficiency. Such interferences have been detected in matrix-assisted laser desorption/ionization (MALDI) mass spectra, where polymer-derived contaminants may obscure or overlap with endogenous peptide peaks (Drake *et al.*, 2004).

Several studies have demonstrated that protein and peptide profiles can vary depending on the type of collection tube used, including differences in clot activators, gel separators, and anticoagulant formulations (Hsieh *et al.*, 2006; Villanueva *et al.*, 2005). These variations may affect protein adsorption to tube surfaces, proteolytic activity, and the recovery of low-abundance biomarkers, thereby compromising reproducibility.

Collection tubes preloaded with standardized anticoagulants and protease inhibitor cocktails have been shown to enhance plasma sample stability and improve reproducibility in quantitative proteomic workflows (Percy *et al.*, 2013). In contrast, commercially available serum collection tubes optimized specifically for high-reproducibility proteomic applications remain limited. Consequently, the Human Proteome Organization (HUPO) has recommended the preferential use of plasma over serum in certain mass spectrometry-based proteomics studies to minimize variability introduced during clot formation and sample processing (Omenn *et al.*, 2004; 2005; 2007; Percy *et al.*, 2013). Overall, careful selection and standardization of blood collection tubes are essential to reduce analytical variability, prevent contamination, and ensure reliability in serum and plasma proteomic investigations.

Sample Stability and Storage

Sample stability is a critical determinant of reproducibility and accuracy in serum and plasma proteomics. Plasma proteins have been reported to exhibit relatively high stability, even after multiple freeze–thaw cycles, with minimal alterations in global protein profiles (Zimmerman *et al.*, 2012; Mateos *et al.*, 2017). This stability supports the suitability of plasma for longitudinal and large-cohort proteomic investigations.

Despite this overall stability, certain circulating proteins demonstrate diurnal variation. Studies have reported circadian fluctuations in proteins such as plasminogen, transthyretin, and apolipoproteins, which are associated with metabolic and regulatory pathways under circadian control (Martino *et al.*, 2007; Robles and Mann, 2013). These findings highlight the importance of standardized sampling time, ideally collecting specimens at consistent times of the day to minimize biological variability (Lundblad, 2003).

In serum samples, proteome variability is closely linked to clotting duration and storage temperature (Hsieh *et al.*, 2006). Apweiler *et al*. (2009) demonstrated that serum peptide profiles can be significantly altered during the clotting process, particularly within 30–60 minutes at room temperature. Extended clotting times may induce ex vivo proteolysis and peptide generation, thereby modifying the detectable proteomic signature.

Temperature control during handling is equally crucial. Experimental evidence indicates that serum samples maintained at room temperature for prolonged periods (e.g., one hour) undergo noticeable proteomic alterations, whereas samples stored on ice during the same interval show minimal changes. Based on HUPO recommendations, serum should be allowed to clot for approximately 60 minutes at room temperature, followed by centrifugation and storage at 4–8 °C for short-term preservation. For long-term storage and to maintain proteome integrity, samples should preferably be stored at −80 °C until analysis (Apweiler *et al.*, 2009). Strict adherence to standardized clotting time, temperature control, and storage conditions is essential to minimize pre-analytical variability and ensure reliable outcomes in

serum- and plasma-based proteomic studies.

Temperature

Temperature control is a critical pre-analytical determinant in maintaining proteome stability and preserving enzymatic activity in serum and plasma samples. As reported for other biological fluids (Greco *et al.*, 2014; Rosenling *et al.*, 2011; Pieragostino *et al.*, 2013), improper temperature regulation can accelerate proteolysis, induce protein denaturation, and alter post-translational modification (PTM) patterns. Therefore, stringent temperature management must be ensured throughout all stages—from sample withdrawal and processing to transport and long-term storage. Several investigations have systematically evaluated the impact of temperature fluctuations on proteomic integrity (Marshall *et al.*, 2003; West-Nielsen *et al.*, 2005).

Rai et al. (2005) suggested that storage in liquid nitrogen represents the optimal condition for preserving protein stability, particularly in large-scale biomarker discovery studies. When liquid nitrogen storage is not feasible, immediate freezing at −80 °C following sample processing is strongly recommended. Samples should preferably be stored in small aliquots to minimize repeated freeze–thaw cycles, which can compromise protein integrity and alter quantitative measurements (Rai *et al.*, 2005; Marshall *et al.*, 2003).

During sample transport and handling, maintenance of the cold chain is essential. Repeated freezing and thawing should be strictly avoided, and refreezing should ideally be limited to no more than two cycles to prevent cumulative proteomic degradation (Rai *et al.*, 2005). In summary, rigorous temperature control at every step of sample handling is fundamental to preserving serum proteome integrity. Each stage—from collection and processing to storage and transport—has a measurable impact on protein stability and analytical reproducibility in proteomic investigations.

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