Heliyon 10 (2024) e40752

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Technical considerations regarding saliva sample collection to achieve comparable protein identification and detection via one- and two-dimensional gel electrophoresis among humans

Szabolcs Maar [a,b,1], Lilla Czuni [b,1], Jørgen Kosberg Hassve [a,b,1], Aniko Takatsy [a,b], Szilard Rendeki [c,d], Tibor Mintal [e], Ferenc Gallyas Jr. [a,b], Ildiko Bock-Marquette [a,b,*]

[a] Department of Biochemistry and Medical Chemistry, University of Pecs, Medical School, Pecs, Hungary
[b] Szentagothai Research Centre, Research Group of Regenerative Science, Sport and Medicine, University of Pecs, Pecs, Hungary
[c] Department of Anesthesiology and Intensive Therapy, University of Pecs, Medical School Pecs, Hungary
[d] Medical Skills Education and Innovation Centre, University of Pecs, Medical School, Pecs, Hungary
[e] Department of Orthopedics, University of Pecs, Medical School, Pecs, Hungary

ARTICLE INFO

Keywords:
Biomarker analysis
Human saliva
Two-dimensional protein electrophoresis
RNA protection

ABSTRACT

Background and aims: Recently, demands towards identifying various molecules in support of stress detection and potential clinical utilization are dramatically increasing. Moreover, the accuracy with which researchers quantify these informative molecules is now far more improved when compared to the past. As RNA or protein markers are conventionally detected via repeated invasive procedures from blood, it is critical to develop secure technologies to obtain the desired information via less stressful methodologies, such as saliva collection. Moreover, for superb interpretation, it became equally significant to obtain the information from the same exact specimen. RNA is easily degradable, thus it is paramount to supplement the samples with protective agents, such as RNAlater, to achieve accurate quantitative results.

Methods: In our research we investigated whether and how this commonly applied RNA protection procedure influences protein and peptide separation of the human saliva via quantitative two-dimensional protein electrophoresis.

Results: Our results revealed, in contrary to previously published data regarding plasma, the addition of RNAlater to saliva samples negatively influences isoelectric focusing and protein detection. We equally found the application oftentimes employed referred to as selective precipitation and reduction-alkylation, partially rescued separation, however, with a significant loss in protein yield and quality when compared to untreated samples.

Conclusion: Our results suggest collection of human saliva for biomarker identification must be performed with extreme diligence. We propose application of RNAlater should be avoided and snap freezing of the collected saliva is recommended when joint protein and RNA quantification is the ultimate goal.

* Corresponding author. University of Pecs, Faculty of Medicine, Department of Biochemistry and Medical Chemistry, Szigeti str.12. Pecs, H-7624, Hungary.
E-mail address: ildiko.bock-marquette@aok.pte.hu (I. Bock-Marquette).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.heliyon.2024.e40752
Received 26 June 2024; Received in revised form 25 November 2024; Accepted 26 November 2024
Available online 28 November 2024

1. Introduction

Environmental stress bears a significant influence on the body resulting in short- and long-term alterations of our physical and mental status. The triggered consequential molecular variations carry invaluable information regarding the state or the potential outcome of these impacts [1]. Doubtlessly, marker molecules which predict diseases or provide status regarding an ongoing action are in the spotlight of today's research, since they are essential for diagnosis and tracing progression, regression, and treatment of certain diseases [2–4]. According to FDA, an ideal marker, also called as biomarker, should be specific for a particular disease and able to differentiate between various physiological states in a safe and easily measurable way [5].

In regard to their nature, biomarkers may consist of a wide range of biochemical and physiological parameters. Vital parameters, such as respiration rate, pulse rate and body temperature, for example, are long term standard measurements at most hospitals, offering a feasible idea of the stress exerted on humans, often supplemented with arterial blood gas for the facile visualization of the internal homeostasis [6]. For more specific visualization, quantified biomarkers in body fluids, such as blood, perspiration or saliva are measured [7]. In many cases however, blood sampling has its drawbacks, including patient compliance, personnel and utility needs, especially when examining children. Doubtlessly, utilizing saliva for these kinds of screening purposes can eliminate the majority of technical and even ethical difficulties [8]. Since salivary glands are surrounded with high permeability capillaries allowing molecules to be exchanged between the capillary blood and the salivary acini, we predict circulating biomarkers can also be present in the excreted saliva [9].

Proteins or smaller secreted peptides are exciting potential biomarker candidates since they are a paramount example of how nature diversifies from one single gene to release multiple, regulated functionalities at the desired sites and time [10–12]. Given the high diversity of peptides in living organisms and their involvement in key regulatory processes, a need for improved peptide discovery, ideally combining peptide sequence identification with peptide profiling, has emerged [13]. The concept includes comprehensive visualization and analysis of small polypeptides, spanning the gap between proteomics and metabolomics [10,14].

Proteins and peptides as biomarkers may be identified or detected by various methods [15–21]. Today, two approaches are commonly utilized to perform proteomic analysis. (1) Bottom-up analysis solubilizes proteins from a sample and is further digested by enzymes or chemicals into peptides [22]. (2) Top-down analysis investigates the intact proteins [23–26]. The bottom-up approach is usually performed when studying complex samples, such as biofluids. Proteins cannot be amplified compared with nucleotides, and bottom-up analysis yields a high resolution of peptides with high sensitivity and with the possibility of improving quantification [22]. The complexity of the proteome, however, is a challenge which inevitably must be encountered, and as formerly explained, separation is a superb means in overcoming these obstacles.

Proteomics relies on three primary technologies: (1) Fractionation, (2) Mass Spectrometry (MS) and (3) Bioinformatics [27]. Tissue or fluid samples typically contain a high complexity of proteins and peptides in a wide range of sizes (1–1.000 kDa). Fractionation allows for the reduction and separation of the molecules. The most utilized (1) gel-based systems separate polypeptides using one- (1DE) or two-dimensional (2DE) polyacrylamide gel electrophoresis to establish a focal point regarding the peptides of interest [28]. These gel-based techniques were developed over forty years ago and are still the most applied separation methods in practice today [28]. One-dimensional polyacrylamide gel electrophoresis was first introduced by Laemmli, in 1970 [29], and the technique separates proteins based on their migratory behavior by applying an electric current. The method provides information regarding the size, purity and relative quantity of the individual proteins [28]. Two-dimensional polyacrylamide gel electrophoresis (first introduced in 1975 by O'Farrell) separates protein samples in two dimensions [30]. The first-dimensional phase separates the proteins based on their isoelectric point (pI value), and the second-dimensional phase separates the proteins based on their molecular weight [30,31]. Since it is implausible in which various proteins may have the same physio-chemical properties, 2DE allows for a more efficient separation of proteins than 1DE. The first dimension, or isoelectric focusing, can be performed conventionally, or using an immobilized pH gradient (IPG). In regards to the conventional method, a carrier ampholyte allows the proteins to migrate until the net charge equals zero, known as their isoelectric point [32]. IPG strips are often pre-made and available in various lengths and pI. The pre-casted acrylamide gel matrix co-polymerized with a pH gradient results in a reasonably stable pH compared with the conventional ampholyte method [33] and has a lower cationic accumulation [32]. The second-dimension separation follows the principles in reference to 1DE and separates the proteins according to molecular weight. Due to simplicity and accuracy, 2DE is an excellent tool for separating potential biomarkers regarding body fluids [34,35]. Visualization is achieved by utilizing staining methods, e.g., Coomassie blue or silver staining. A high accumulation of color in one region implies a high abundance of similar proteins and is typically excised from the gel and enzymatically digested by trypsin. The proteins are next analyzed by MS (2) or tandem MS to identify the proteins [36]. MS measures the mass-charge ratio of ions and is capable of determining sequence information to precisely identify the examined peptide [37,38]. Bioinformatics (3) allows for rapid identification of the information obtained from MS through several libraries of software packages [36].

To retrieve comparable quantitative results regarding biomarker identification and analysis via high sensitivity instrumentation and methodologies, it is equally imperious to devote immense attention towards the technical details of sample collection. Improper sample handling may result in significant output alterations leading to false high throughout data [39,40]. First, the procedure must be prompt, none-invasive, causing minimal or no pain and infection, especially when approaching children. Recently, due to COVID pandemics, numerous methods and technologies have been proposed to gain admissible quality of bio-fluids, especially saliva [40–43]. The descriptions, however, focus either on RNA or protein retrieval independently. To achieve admissible data for biomarker identification and detection, the most prosperous approach is to extract both RNA and protein molecules from the selfsame sample pool. Without an option for snap freezing (e.g., as in field collection), due to intense RNase activity in the saliva, it is requisite to employ protection at the time of collection [39,44]. RNAlater^{TM} solution (Thermo Fisher) is often utilized to overcome these obstacles. As

recommended by the user's technical support (Thermo Fisher, RNA isolation, TechNotes), in addition to high-quality RNA yield, intact protein can be recovered from RNAlater-stabilized samples for use in downstream applications such as Western blotting or 2D gel analysis [45]. However, the results of these protocols were only collected by utilizing stabilized mammalian tissue samples. Thus, since saliva sampling is one of the best non-invasive methodologies for bio-marker analysis and screen, and RNA and protein results are most reliable and comparable when achieved from the same sample pool, we asked whether direct application of RNAlater™ to saliva samples remains an equally safe practice to restore RNA and protein content for RNA sequencing, Western blotting or 2D gel and Mass Spec analyses. In our study we performed SDS-page electrophoresis and 2D gel electrophoresis investigations utilizing human whole saliva samples collected via spitting method [46] with or without RNAlater™, respectively, and compared the quality and quantity of protein retrieval.

2. Materials and methods

2.1. Materials

All experiments were performed utilizing low protein binding Eppendorf tubes (Eppendorf) and low protein binding pipette tips (Eppendorf). All materials were MS grade. Urea, glycerol and mineral oil were obtained from Sigma, thiourea, sodium thiosulfate, bromophenol blue, glycine from Reanal, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), Bio-Lyte, dithiothreitol, iodoacetamide, Silver Stain Plus Kit and Bio-Safe Coomassie G-250 Stain from Bio-Rad, and SDS from Serva. Silver nitrate was purchased from Hungaropharma, acetic acid and formaldehyde from Molar Chemicals, methanol from Merck LabGrade, and sodium bicarbonate from Acros Organics.

2.2. Ethics and consent/sample collection

Prior inception regarding our study, an ethical approval was obtained from the Regional and Institutional Research Ethical Committee of the University of Pecs (Protocol number: KK6155) (Issued: April 22, 2017). All experimental procedures fully conformed with the National Health and Medical Research Council Guidelines for Experimentation with Human Subjects. Volunteers were recruited for a period of three days. Prior to participation, all volunteers were informed concerning the aims of the study and provided with both information and a form for their participatory consent in writing. The privacy rights of human subjects were always and strictly observed. No minor volunteers were participating in this presented study.

One ml of saliva samples from three adult healthy volunteers (two female, one male; age 25–28) were collected via spitting method [46] at identical times in the morning, prior to drinking or eating. RNAlater™ (Thermo Fisher) or dH_2O was added to the saliva samples in a 1:1 ratio immediately following collection, respectively. Next, samples were briefly centrifuged at 13,500 rpm for 10 min at 4 °C to pellet any insoluble material. Supernatants were aliquoted and stored at −80°C until further processing and analysis.

2.3. One dimensional gel electrophoresis (1DE)

Saliva samples were thawed on ice and centrifuged at 13,500 rpm for 10 min at 4 °C. Samples were mixed in 1:1 ratio with Laemmli buffer containing 5 % of β-mercaptoethanol (Bio-Rad) and incubated at 95 °C for 15 min. Following denaturation, 15 μl of the total sample volume was loaded onto 12 % polyacrylamide gels for silver, and 30 μl for Coomassie staining (Coomassie Brilliant Blue R-250 (Bio-Rad)), respectively. Separation was carried out utilizing Mini-Protean Tetra cell® (Bio-Rad) chamber and PowerPac™ (Bio-Rad) power supply with a constant current of 7.5mA/gel for stacking and 15 mA/gel for resolving and separation. The 30 μl protein gels were stained via Coomassie blue overnight at room temperature via gentle rocking (Colloidal Coomassie R-250 Bio-Rad) followed by a destaining step utilizing 10 % acetic acid, 50 % methanol and 40 % H_2O. In support of enhancing protein visualization via silver staining, the 15 μl protein containing gels were fixed in 10 % acetic acid, 50 % methanol, and 40 % H_2O for 20 min and incubated in 50 % methanol for 2 × 10 min at room temperature, respectively. Next, gels were rinsed with H_2O for 10 min, which was followed by a sensitization utilizing 0.2 % $Na_2S_2O_3$ solution for 1 min. Following sensitization, gels were rinsed with ddH_2O for 2 × 10 min and incubated in 0.2 % $AgNO_3$ solution for 20 min at 4 °C followed by 2 × 1 minute washing with ddH_2O. Color development was performed using 0.04 % formaldehyde/2 % Na_2CO_3 solution until desired coloration and the reaction was stopped via 3 % acetic acid for 5 min. Following termination, gels were washed with ddH_2O for 5 min and stored in 1 % acetic acid at 4 °C.

2.4. Protein preparation

In consideration of protein extraction and improved visualization, we acquired ReadyPrep™ 2-D Cleanup kit (Bio-Rad). To decrease horizontal striking ReadyPrep™ Reduction-Alkylation kit (Bio-Rad) was equally utilized. Both kits were applied in full accordance to the manufacturer's recommended guidelines.

2.4.1. ReadyPrep™ 2-D cleanup kit

First, protein samples were transferred into microcentrifuge tubes, then 300 μl precipitating agent 1 (Bio-Rad) was added and incubated for 15 min on ice. Following incubation 300 μl precipitation agent 2 (Bio-Rad) was applied and centrifuged for 5 min at 12,000 rcf to form a pellet. The supernatant was removed and discarded followed by a 15–30 s centrifugation to collect and discard residual liquid. Following the addition of 40 μl wash reagent 1 (Bio-Rad) to the pellet samples, the mixture was centrifuged at 12,000

rcf for 5 min at room temperature. Next, a wash reagent was discarded followed by the addition of 25 μl ultrapure water to the pellets by vortexing for 10–20 s. Next, 1 ml wash reagent 2 (Bio-Rad) and 5 μl of wash 2 (Bio-Rad) was added, respectively, vortexed and incubated for 30 min at −20 °C. During incubation, the mixture was vortexed for 30 s every 10 min. Following incubation, the tubes were centrifuged at maximum speed (14,000 rcf) for 5 min to form a tight pellet. Lastly, the supernatant was removed. The pellet was air-dried and finally resuspended in 2D rehydration buffer (Bio-Rad).

2.4.2. ReadyPrep™ Reduction-alkylation kit

15 mM iodoacetamide solution was prepared immediately prior to utilization. Following protein sample preparation in rehydration buffer, the pH of each protein sample was adjusted to 8.0 and 9.0 via alkylation buffer, respectively. Next, tributyl-phosphine (TBP) was added for a final concentration of 5 mM and incubated for 30 min at room temperature. Following incubation, iodoacetamide was added to the samples to achieve a final concentration of 15 mM and further incubated for 1 h at room temperature. Following incubation, TPB was added to quench any unreacted iodoacetamide, incubated for 15 min at room temperature and centrifuged at 16,000 rcf for 5 min to pelletize any insoluble material. Finally, protein concentration was adjusted to a detectable level via rehydration buffer (Bio-Rad) and samples were loaded onto IPG strips.

2.5. Two-dimensional gel electrophoresis

In consideration of isoelectric focusing, saliva samples were thawed on ice and briefly centrifuged at maximum speed at 4^{o}C prior to loading onto 7 cm pH 3–10 Non-Linear (NL) ReadyPrep IPG (Immobilized pH Gradient) strips (Bio-Rad), respectively. In our experiments, we utilized NL strips since they permit high resolution separation at the central pH range (pH 5–8). IPG strips were next rehydrated in a rehydration buffer solution containing 7M Urea, 2M Thiourea, 4 % 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) and 0.001 % Bromophenol blue. Aliquots of the buffer solution were stored at −20 °C. First, 50mMDTT was added to the rehydration buffer and protein mixture. The samples were vortexed and incubated for 15 min at 37 °C. Next, 0.5 % Ampholyte (Bio-Rad, Bio-Lyte pH 3.6–9.5) was added, vortexed and incubated for 30 min at 37 °C to each sample, respectively. The solutions were then centrifuged at 16,000 rcf for 10 min at room temperature to remove any insoluble material. Subsequently, 125 μl of the supernatant was evenly loaded to separated lanes. The strips were placed into the protein solution and incubated for 30 min at room temperature. Next, 3 ml mineral oil was added to each strip, covered, and stored for 16 h at room temperature (passive rehydration). Following passive rehydration, the rehydrated strips were positioned into a focusing tray. The electrodes, retainers and lanes of the 7 cm focusing tray were thoroughly cleaned using 96 % ethanol. 2.5 ml mineral oil was repeatedly added to the strips to further prevent evaporation and overheating and then inserted into an isoelectric focusing device (Bio-Rad Protean i12 IEF Cell). To avoid horizontal streaking, the focusing protocol was altered, per the supplier's recommendations, of which, follows. Part one: 50V for 15 min followed by 100V linear ramping for 1 h. Next, the paper wicks were exchanged and continued forward with Part two: 1. 50 V for 15 min followed by 2. three linear gradients. *a.* 50–100 V for 15 min, *b.* 100–150 V in 15 min and *c.* 150–250 V for 15 min. 3. linear voltage ramp to reach 4000 V for 2 h, 4. linear gradient ramp to reach 10000 V/h limit, 5. 500 V hold for unlimited time. Next, focusing strips were removed and placed into a clean rehydration/equilibration tray and stored at −80 °C to preserve separation quality. The strips were stored at −80 °C for several months until further application. Next, 12 % polyacrylamide gels were utilized for further SDS-PAGE separation of the focused proteins. To reduce disulfide bridges for a refined separation, strips from the first-dimensional separation were equilibrated in a total volume of 5 ml of buffer mixes (2.5 ml of Buffer I and 2.5 ml of Buffer II). Buffer I: 6M urea, 0.2 % SDS, 20 % glycerol, 0.375M Tris-HCl pH 8.8 including 130 mM DTT directly, prior to utilization of the buffer mix. Buffer II: 6M urea, 0.2 % SDS, 20 % glycerol, 0.375M Tris-HCl pH 8.8 including 135 mM iodoacetamide directly prior to utilization of the buffer mix. To achieve second-dimensional separation, proteins were further separated via 20 mA constant current according to their molecular weight utilizing Protean II xi Cell System (Bio-Rad) next to All Blue Standard (Bio-Rad) molecular weight marker in Tris-Glycine SDS running buffer at 4 °C overnight. Separation was terminated once the frontline reached 1 cm distance from the bottom of the gel.

2.6. Protein staining and evaluation

To enhance visualization of the separated peptides and proteins, Mass Spec compatible bio-safe silver staining (Bio-Rad) was performed in full accordance with the manufacturer's recommended protocol [47]. Shortly, following the addition of 50 % Methanol (MeOH) and 50 % glacial acetic acid (CH_3COOH), the mixture was fixated for 30 min at room temperature, gels were washed twice in 50 % MeOH and once in ion-exchanged water for 10 min. Sensitization in 0.2 % sodium thiosulfate 5-hydrate ($Na_2S_2O_3$) was performed for 1 min, followed by a rinse in water for 10 min at room temperature, respectively. Next, the gels were incubated in silver nitrate ($AgNO_3$) for 20 min at 4 °C, followed by a 2 × 1 minute rinsing step in ddH_2O. Development was performed utilizing 0.04 % formaldehyde in 2 % Sodium carbonate (Na_2CO_3). The rection was terminated with 3 % acetic acid followed by a rinse in water for 5 min. Gels were then stored in 1 % acetic acid at 4 °C until further processing.

2.7. Gel documentation

All gels were documented via ChemiDoc™ XRS + system (Bio-Rad), images were captured utilizing Image Lab™ Software.

3. Results

3.1. RNAlater™ treatment of human saliva samples significantly alters protein separation via SDS-PAGE

To fulfil our intention to investigate the alterations of potential biomarkers in human saliva, we first initiated SDS-PAGE separation following RNAlater™ treatment. To ensure equal input for each protein sample, we first measured protein concentrations of the saliva via Qubit™ protein assay (Life Technologies). We found RNAlater™ treated samples offer scattered protein concentrations compared to untreated saliva samples utilizing Qubit™ protein assay rendering accurate protein input characterization nearly impossible (data not shown). To circumvent this obstacle, we utilized equivalent input volumes of saliva, in which modifying agents were substituted by equal volumes of distilled water. Next, we investigated RNAlater™ treated and untreated saliva samples via one-dimensional SDS polyacrylamide gel electrophoresis (SDS-PAGE). As demonstrated in Fig. 1, in contrast to native samples, saliva proteins containing RNAlater™ were equally under-represented during one-dimensional gel electrophoresis, in particular, at the low molecular range (Fig. 1, Lanes 3–4). Moreover, we discovered, independently of the input concentration, the quality of untreated sample separation was significantly different (Fig. 1, Lanes 1–2) when compared to those with RNAlater™ (Fig. 1, Lanes 3–4), indicating the addition of RNAlater™ interferes with one-dimensional SDS-PAGE separation of saliva proteins. Additionally, our experiments confirmed, the sensitivity of silver staining is superior to Coomassie staining, even when the later contained twice of the loading volume (15 μl). Given these results, we preferred utilizing silver staining in future analyses (Fig. 1b).

3.2. Application of RNAlater™ impairs 2D protein separation

Due to our controversial results regarding SDS page, we tested whether and how RNAlater™ may impact two-dimensional gel electrophoresis separation of the saliva proteins. During separation, a constant maximum current limit was set to 50μA, since further elevation generates increased heat load and consequential melting of the strips during separation. First, we performed isoelectronic focusing (IEF) of the treated and untreated samples, respectively. As presented in Fig. 2, IEF utilizing RNAlater™ treated samples revealed incomplete (Fig. 2b and c). Live analysis regarding the current and voltage patterns of isoelectric focusing indicated, voltage levels did not reach the required values when processing RNAlater™ containing saliva samples (Fig. 2c, blue arrowhead) most likely due to the downregulating effect of the successively elevated current, a potential consequence of increased salt concentrations in our samples (Fig. 2c, blue arrow). Notably, simultaneous isoelectric focusing of untreated samples did not indicate improper separation (Fig. 2c, green arrowhead, green arrow). Moreover, our results equally revealed, RNAlater™ does not impact second step size separation when compared to untreated samples (Fig. 2a and b). Finally, and controversially, our data implicate the total quantity of the proteins during second step SDS-PAGE remained visibly unaltered when compared to the results regarding SDS-PAGE, without initial IEF, suggesting a potential general loss of total protein content during IEF (Fig. 1).

3.3. Impact of potential "rescue" procedures on 2D separation yield and quality

To overcome the aforementioned obstacles, our next goal was to identify methodologies which may eliminate or reduce the hindering impact of RNAlater™ on saliva IEF during 2D separation. Due to the suspected increase in ionic contaminants of our samples, we utilized selective precipitation (ReadyPrep 2-D Cleanup kit™; Bio-Rad (CU)) with the additional application of reduction-alkylation (ReadyPrep Reduction-Alkylation kit™; Bio-Rad (RA)) [48] prior IEF procedures to achieve a preferable separation and yield. First, we investigated whether the collective utilization of the aforementioned procedures possibly bear any impact upon untreated samples. Our results revealed the joint application of both procedures significantly alters protein yield, especially at the lower molecular range (Fig. 3, Areas 1–3).

According to published results, the reduction-alkylation of protein samples decreases horizontal striking and accelerates separation

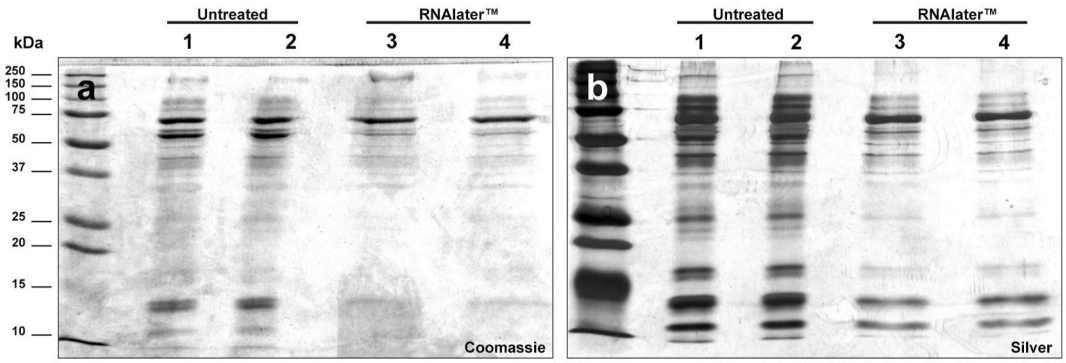

Fig. 1. RNAlater™ treatment alters protein separation via SDS-PAGE. Representative one-dimensional SDS-PAGE results of untreated (Lanes 1–2) and RNAlater™ treated (Lanes 3–4) human saliva samples followed by (**a**) Coomassie staining and (**b**) Silver staining demonstrate decrease in protein yield when RNAlater™ was applied. MWM: molecular weight marker; kDa: kilodaltons.

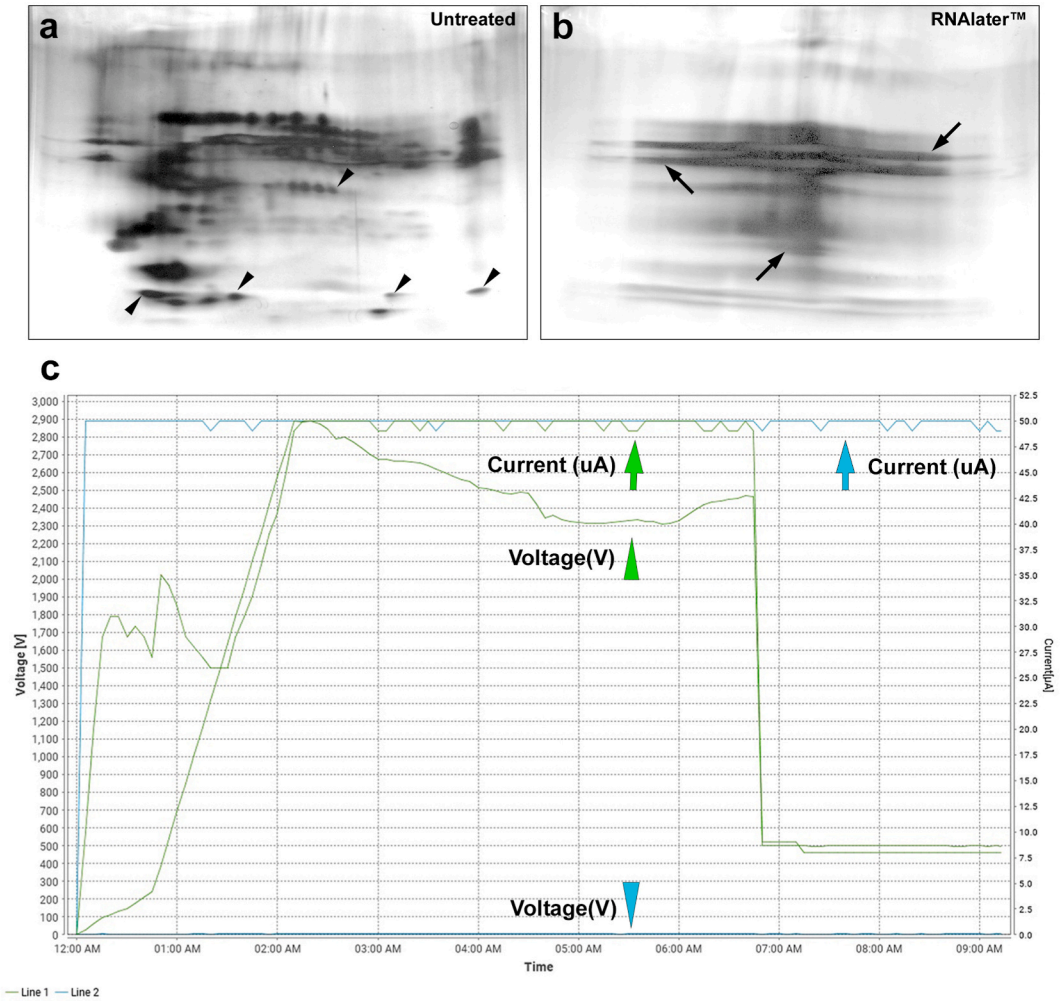

Fig. 2. RNAlater™ treatment influences 2D gel electrophoresis separation of saliva proteins in a negative manner. a, b, Representative two-dimensional gel electrophoresis results of untreated (**a**) saliva and RNAlater™ treated (**b**) human saliva samples via silver staining reveal impaired separation of saliva proteins. Arrowheads point at independent protein spots; arrows indicate compromised isoelectric focusing. (**c**) Representative chart of 2D isoelectric separation displaying RNAlater™ (blue) significantly alters voltage (arrowheads) and current (arrows) patterns during separation when compared to non-treated (green) saliva samples.

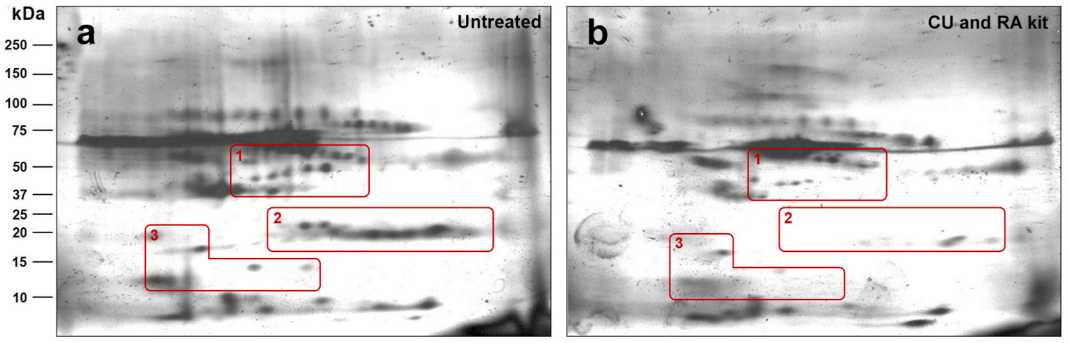

Fig. 3. Utilization of Bio-Rad ReadyPrep 2-D Cleanup kit™ and ReadyPrep Reduction-Alkylation kit™ on native human saliva samples alter protein yield. Representaive 2D gel electrophoresis results of untreated (**a**) and ReadyPrep 2-D Cleanup kit™, ReadyPrep Reduction-Alkylation kit™ treated (**b**) native human saliva samples (no RNAlater™ treatment) demonstrate both procedures alter protein yield primarily at the low molecular range. Highlighted areas (1–3) indicate identical regions of the separation. Proteins were visualized by silver stain.

during isoelectric focusing primarily by inhibiting reconstitution of disulfide bounds between the molecules [48]. Thus, we investigated whether the reduction-alkylation procedure itself may bear any influence on quantitative 2D protein detection when applied together with protein clean-up. Notably, the utilization of the Cleanup kit™ was critical in our setting, since without selective precipitation, IEF was unimplementable when using saliva with RNAlater™ (Fig. 2b). Our results confirmed the use of Reduction-Alkylation kit™ slightly improves spot detection and resolution (Fig. 4, Black arrowheads).

Next, because we were unable of achieving isoelectric focusing on samples treated with RNAlater™ (with no other additional treatment) during our experiments, we compared RNAlater™ containing precipitated and reduction-alkylated human saliva with native, untreated samples (Fig. 2b). Our results revealed, the utilization of ReadyPrep 2-D Cleanup kit™ and ReadyPrep Reduction-Alkylation kit™ enabled isoelectric focusing, although the loss of protein yield became considerably decreased, thus inapplicable regarding experiments for quantitative utilization (Fig. 5).

Since the reduction-alkylation resulted in a visible increase regarding separation quality and yield in comparison to Cleanup kit™ treated native saliva (Fig. 4), we asked whether and how the procedure influences isoelectric focusing of saliva samples with RNAlater™. We found that utilization of ReadyPrep Reduction-Alkylation kit™ may improve 2D separation for some target proteins, however, the overall changes were less striking and significant when compared with the results with no RNAlater™ (Figs. 4 and 6).

Finally, our investigations revealed that even if saliva samples are treated with the same protein precipitation and reduction-alkylation kits, RNAlater™ treatment significantly reduces protein yield and spot resolution when compared to samples without the addition of the compound (Fig. 7.).

4. Discussion

Stress is a persistent factor significantly affecting the entirety of the human body, which oftentimes results in irreversible pathological alterations [49]. Early detection of the warning signs is critical and may save many lives. Recently, the identification of stress and/or disease specific molecules, so-called biomarkers, received significant attention [1,3,4,6,11,19,21]. Monitoring stress impact and consequential diseases may be equally utilized among the general population, thus providing novel models for targeted therapy [1,3,4,12,15,21,37]. Currently, proteomics is a dynamically emerging scientific field in which discovering novel biomarkers regarding physical or psychological stress can potentially be effectively determined [50–52]. Biomarkers may be generally collected from various body fluids, such as blood, urine, perspiration, tears or saliva [2,11,16,20,26,53,54]. Well established protocols regarding stress biomarker detection in the plasma already demonstrated excellent results [11,55]. Naturally, one intends to utilize a similar regime for saliva analysis, as contrary to blood pooling, one of the advantages of saliva collection is the lack of invasive canulation requirements. Moreover, salivary biomarkers recently emerged as a new and widely researched field [56–58]. Thus, in addition to mRNA and miRNA, comparing the extensively researched plasma proteome to the salivary profile, we intended to develop a sophisticated protocol for synchronous sampling and analysis to avoid the burden of venous sampling in the future.

Saliva possesses a large number of proteins and RNA, yet it also contains considerable amounts of non-human RNA and endonucleases [59]. To ensure proper stabilization and protection of the RNA pool, a commonly utilized process is to include protective agents such as RNAlater™ to our sampling [39,40,44]. Based on numerous data, RNAlater™ proves to be an outstanding tool when acquiring on-field samples, yielding high quantity and quality RNA collected from various body fluids [43]. Circulating miRNAs are emerging as valuable biomarkers for various physical and psychological conditions and regulate protein levels by directly interconnecting with their targeted mRNA at its 3′UTR region to primarily inhibit post-transcriptional translation [60,61]. Thus, miRNA levels and protein alterations are firmly linked, since they may also reflect the physical and psychological state of the human body. Bearing these facts in mind, the ultimate aim is to synchronously investigate the expressed small proteins and miRNAs in regards of the collected saliva samples. To successfully achieve this goal, it is absolutely critical RNAlater™ will not damage or influence salivary peptide detection.

Without knowing the exact composition of the utilized RNAlater™ (Thermo Fisher), since it is a trade secret, RNA stabilizing agents

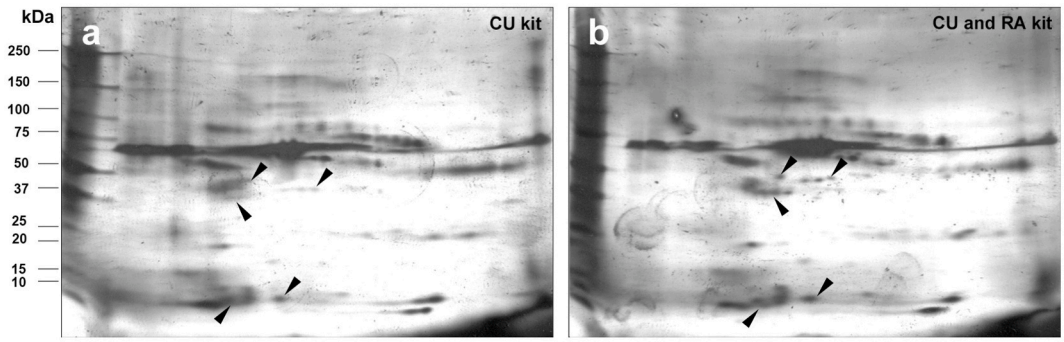

Fig. 4. Impact of reduction-alkylation during 2D gel electrophoresis of native saliva samples. Representative 2D gel electrophoresis results on human saliva samples with the use of ReadyPrep 2-D Cleanup kit™ (**a**) in comparison with ReadyPrep 2-D Cleanup kit™ and ReadyPrep Reduction-Alkylation kit™ (**b**) treated samples indicate Reduction-Alkylation kit™ slightly improves spot detection and resolution. Arrowheads highlight visible differences in spot resolution and protein yield quality.

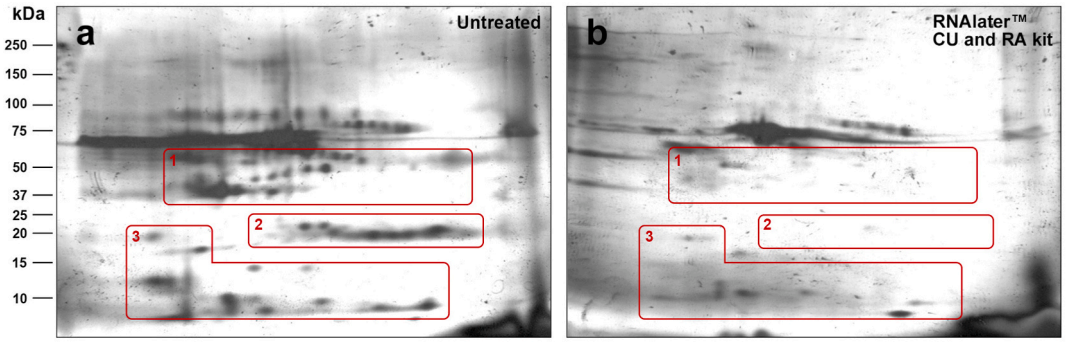

Fig. 5. Altered protein yields of RNAlater™ containing saliva samples following ReadyPrep 2D Cleanup kit™ and ReadyPrep Reduction-Alkylation kit™ treatments in comparison with untreated, native saliva samples. 2D gel electrophoresis results of a representative untreated human saliva (**a**) compared with RNAlater™, ReadyPrep 2-D Cleanup kit™ and ReadyPrep Reduction-Alkylation kit™ treated sample (**b**) indicate a significant decrease in protein yields despite of the protective manipulations. Highlighted areas (1–3) represent identical protein regions following separation.

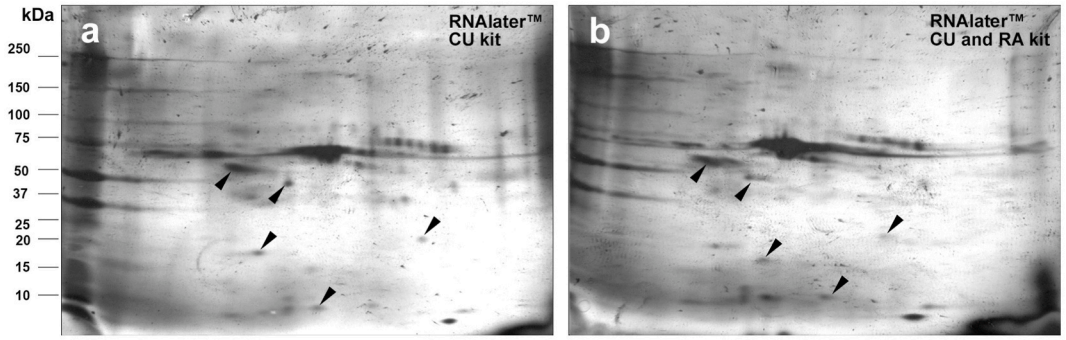

Fig. 6. Application of ReadyPrep Reduction-Alkylation kit™ does not improve the quality 2D gel electrophoresis of human saliva samples when pretreated with RNAlater™. Representative 2D gel electrophoresis of human selectively precipitated saliva samples with RNAlater™ (**a**) in relation with saliva sample treated with RNAlater™, ReadyPrep 2-D Cleanup kit™ and ReadyPrep Reduction-Alkylation kit™ (**b**) demonstrate lack of significant separation improvement with RA (Black arrows point at identical proteins on a and b).

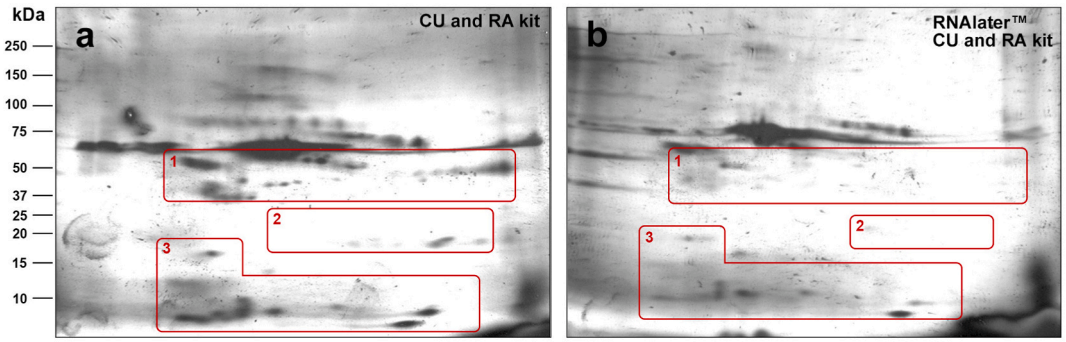

Fig. 7. Impact of RNAlater™ on 2D gel electrophoresis of saliva samples treated with both, ReadyPrep 2-D Cleanup kit™ and ReadyPrep Reduction-Alkylation kit™. The addition of RNAlater™ to precipitated and reduction-alkylated protein samples demonstrates a significant reduction of protein yield and quality following 2D separation (**b**) when compared to saliva without RNAlater™ (**a**). Numbers 1–3 correspond to similar regions of interest.

typically consist of ammonium sulphates and caesium sulphate, which may denature proteins [62]. Bennike et al. demonstrated by utilizing a modified FASP tryptic protein digestion protocol for sample preparation, the intestinal tissue samples may be stored in RNAlater with minimal impact upon proteome results [62]. Moreover, Espinosa-de Aquino et al. showed how mucosal swabs, obtained from sea lions and treated with RNAlater, led to the peptide profile being detectable, using SDS-PAGE and western blot [63]. In their experiments, they utilized TRIzol™ for RNA isolation and concurrent protein precipitation from the same sample. The disadvantage of

this technique is its inappropriateness for precise quantitative investigations, particularly when identifying a low molecular weight protein profile, which is often our intent. In this present study, we investigated human saliva samples, with or without the addition of RNAlater™ (Thermo Fisher), with an ultimate goal to precisely determine the influence regarding this regularly employed RNA protection solution on 2D protein separation and sequencing quality.

In our research, the addition of RNAlater™ to saliva samples not only decreased but even disabled peptide separation and detection following one-dimensional (Fig. 1), or two-dimensional electrophoresis (Fig. 2). Disregarding the solution resulted in comparable images and separation quality (Fig. 3), indicating RNAlater™ may not be suitable for preserving secreted samples for proteomic studies, as others, with success, utilized cell-rich tissues [62,63]. Among the potential alternatives, we rejected the use of TRIzol™ since our experience resulted in interference with protein detection by the loss of smaller-sized molecules during precipitation of the protein fraction (data not shown). Rather, as an alternative approach to remove RNAlater™ from our biofluids, we conducted our experiments using ReadyPrep 2-D cleanup kit™ and/or ReadyPrep reduction-alkylation kit™. Cleanup removes ionic contaminants, which may interfere with separation in IEF and 2-DE. ReadyPrep Reduction-Alkylation kit™ blocks the formation of disulfide bonds prior to isoelectric focusing. Through our experiments we found utilization of ReadyPrep Reduction-Alkylation kit™ (along with ReadyPrep 2-D Cleanup kit™) in native samples seemed beneficial for 2D gel electrophoresis since it improved spot detection and slightly reduced horizontal striking in our gels (Fig. 4). As represented in Fig. 6, the joint addition of Cleanup kit™ and Reduction-Alkylation kit™ to RNAlater™ treated samples equally provided some improvement in separation quality, however, the achieved results were far not comparable with non-RNAlater™ treated samples (Figs. 5 a and Fig. 7), especially at the low molecular weight range. Comprehensively, these results indicated the application of the utilized “rescue procedures” on RNAlater™ treated saliva samples becomes only partially suitable, especially when precise comparison of protein alterations prior to and following physical or psychological stress is our goal. Moreover, RNAlater™ treated saliva sample alterations did not resemble the results acquired from plasma, emerging as a significant barrier in comparing protein expression of the two biofluids. We equally determined, similar to TRIzol™ purification, precipitation and reduction-alkylation equally restrict sample analysis and comparison by excluding low molecular weight peptides off the screen.

In summary, contrary to previously published recommendations regarding cell-rich tissue samples [62,63], our results suggest proper saliva sample collection should be performed without utilizing RNAlater™ protection solution when performing proteomic studies. Instead, immediately snap-freezing the specimens in liquid nitrogen or dry ice/ethanol is recommended to achieve quantitative and comparable RNA/protein results for potential biomarker identification or detection. Moreover, we demonstrated the application of Cleanup™ and Reduction-Alkylation™ kits are to be equally avoided when rigorous quantitative protein separation and identification is our experimental goal.

CRediT authorship contribution statement

Szabolcs Maar: Writing – original draft, Visualization, Investigation. **Lilla Czuni:** Methodology, Investigation. **Jørgen Kosberg Hassve:** Writing – original draft, Visualization, Formal analysis. **Aniko Takatsy:** Supervision, Methodology. **Szilard Rendeki:** Resources, Funding acquisition. **Tibor Mintal:** Resources. **Ferenc Gallyas:** Supervision. **Ildiko Bock-Marquette:** Writing – review & editing, Funding acquisition, Conceptualization.

Ethics statement

Ethical approval was obtained from the Regional and Institutional Research Ethical Committee of the University of Pecs (Protocol number: KK6155) (Issued: April 22, 2017). All experimental procedures were fully performed in compliance with the relevant laws and institutional guidelines of the National Health and Medical Research Council of Hungary for Experimentation with Human Subjects. The work presented has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. The privacy rights of human subjects were always and strictly observed.

Data and code availability

Data will be made available upon request.

Funding

This work was supported by grants from OTKA-K108550, GINOP-2.3.2-15-2016-00047 of IBM, by TKP2021-NVA-06, which has been implemented with the support provided from the National Research, Development and Innovation Fund of Hungary financed under the TKP2021-NVA funding scheme, and by 2022–2.1.1-NL-2022-00012 ″National Laboratory of Cooperative Technologies", provided by the Ministry of Culture and Innovation from the National Research, Development and Innovation Fund of Hungary financed by the National Laboratories program of SzR.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgements

The authors express their sincere gratitude to Jon E. Marquette for reviewing and editing their manuscript, and to Helena Halasz for her technical support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e40752.

References

[1] B. Mayer, et al., Predictive biomarkers for linking disease pathology and drug effect, Curr Pharm Des 23 (1) (2017) 29–54.
[2] C. Backes, E. Meese, A. Keller, Specific miRNA disease biomarkers in blood, serum and plasma: challenges and prospects, Mol. Diagn. Ther. 20 (6) (2016) 509–518.
[3] P.F. Huesgen, P.F. Lange, C.M. Overall, Ensembles of protein termini and specific proteolytic signatures as candidate biomarkers of disease, Proteomics Clin Appl 8 (5–6) (2014) 338–350.
[4] J.L. Vincent, E. Bogossian, M. Menozzi, The future of biomarkers, Crit. Care Clin. 36 (1) (2020) 177–187.
[5] K.K. Jain, The Handbook of Biomarkers, second ed., xiv, Humana Press, New York, NY, 2017, p. 760.
[6] O.P. James, et al., Biosensors, biomarkers and biometrics: a bootcamp perspective, BMJ Simul Technol Enhanc Learn 7 (4) (2021) 188–193.
[7] K. Dhama, et al., Biomarkers in stress related diseases/disorders: diagnostic, prognostic, and therapeutic values, in: Frontiers in Molecular Biosciences, 2019, p. 91.
[8] K. An, et al., Salivary biomarkers of chronic psychosocial stress and CVD risks: a systematic review, Biol. Res. Nurs. 18 (3) (2016) 241–263.
[9] C.Z. Zhang, et al., Saliva in the diagnosis of diseases, Int. J. Oral Sci. 8 (3) (2016) 133–137.
[10] P. Schulz-Knappe, M. Schrader, H.D. Zucht, The peptidomics concept, Comb. Chem. High Throughput Screen. 8 (8) (2005) 697–704.
[11] P.E. Geyer, et al., Revisiting biomarker discovery by plasma proteomics, Mol. Syst. Biol. 13 (9) (2017) 942.
[12] S. Hanash, Disease proteomics, Nature 422 (6928) (2003) 226–232.
[13] K. Suhre, M.I. McCarthy, J.M. Schwenk, Genetics meets proteomics: perspectives for large population-based studies, Nat. Rev. Genet. 22 (1) (2021) 19–37.
[14] B. Domon, R. Aebersold, Mass spectrometry and protein analysis, Science 312 (5771) (2006) 212–217.
[15] E.S. Boja, H. Rodriguez, The path to clinical proteomics research: integration of proteomics, genomics, clinical laboratory and regulatory science, Korean J Lab Med 31 (2) (2011) 61–71.
[16] O. Chertov, et al., Enrichment of low-molecular-weight proteins from biofluids for biomarker discovery, Expert Rev. Proteomics 2 (1) (2005) 139–145.
[17] Y.T. Cho, et al., Biomarker characterization by MALDI-TOF/MS, Adv. Clin. Chem. 69 (2015) 209–254.
[18] L. Denoroy, et al., Ultra high performance liquid chromatography as a tool for the discovery and the analysis of biomarkers of diseases: a review, J Chromatogr B Analyt Technol Biomed Life Sci 927 (2013) 37–53.
[19] P.Y. Lee, et al., Plasma/serum proteomics: depletion strategies for reducing high-abundance proteins for biomarker discovery, Bioanalysis 11 (19) (2019) 1799–1812.
[20] S. Mahboob, et al., Is isolation of comprehensive human plasma peptidomes an achievable quest? J Proteomics 127 (Pt B) (2015) 300–309.
[21] U. Rajcevic, S.P. Niclou, C.R. Jimenez, Proteomics strategies for target identification and biomarker discovery in cancer, Front Biosci (Landmark Ed) 14 (9) (2009) 3292–3303.
[22] V.A. Duong, H. Lee, Bottom-up proteomics: Advancements in sample preparation, Int. J. Mol. Sci. 24 (6) (2023).
[23] K.A. Cupp-Sutton, S. Wu, High-throughput quantitative top-down proteomics, Mol Omics 16 (2) (2020) 91–99.
[24] J.L. Nickerson, et al., Recent advances in top-down proteome sample processing ahead of MS analysis, Mass Spectrom. Rev. 42 (2) (2023) 457–495.
[25] A. Tholey, A. Becker, Top-down proteomics for the analysis of proteolytic events - methods, applications and perspectives, Biochim. Biophys. Acta Mol. Cell Res. 1864 (11 Pt B) (2017) 2191–2199.
[26] Z. Zhang, et al., High-throughput proteomics, Annu. Rev. Anal. Chem. 7 (2014) 427–454.
[27] R. Aebersold, M. Mann, Mass-spectrometric exploration of proteome structure and function, Nature 537 (7620) (2016) 347–355.
[28] H. Husi, A. Albalat, in: S.B.T.-H.o.P.a.S.M. Padmanabhan (Ed.), Chapter 9 - Proteomics, Academic Press, San Diego, 2014, pp. 147–179.
[29] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature (1970) 680–685.
[30] P.H. O'Farrell, High resolution two-dimensional electrophoresis of proteins, J. Biol. Chem. (1975) 4007–4021.
[31] N. Saraswathy, P. Ramalingam, 10 - Introduction to proteomics, in: N. Saraswathy, P. Ramalingam (Eds.), Concepts and Techniques in Genomics and Proteomics, Woodhead Publishing, 2011, pp. 147–158.
[32] S. Magdeldin, Gel Electrophoresis: Principles and Basics, BoD–Books on Demand, 2012.
[33] S. Magdeldin, et al., Basics and recent advances of two dimensional- polyacrylamide gel electrophoresis, in: Clinical Proteomics, Springer, 2014, p. 16.
[34] M.-R. Kim, C.-W. Kim, Human blood plasma preparation for two-dimensional gel electrophoresis, in: Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences, Elsevier B.V., 2007, pp. 203–210.
[35] A. Rogowska-Wrzesinska, et al., 2D gels still have a niche in proteomics, J Proteomics 88 (2013) 4–13.
[36] L.-R. Yu, N.A. Stewart, T.D. Veenstra, in: G.S. Ginsburg, H.F.B.T.-E.o.G.a.P.M. Willard (Eds.), Chapter 8 - Proteomics: the Deciphering of the Functional Genome, Academic Press, San Diego, 2010, pp. 89–96.
[37] I.A. Brewis, P. Brennan, Proteomics technologies for the global identification and quantification of proteins, Adv Protein Chem Struct Biol 80 (2010) 1–44.
[38] K. Gevaert, J. Vandekerckhove, Protein identification methods in proteomics, Electrophoresis 21 (6) (2000) 1145–1154.
[39] Y. Nishibata, et al., RNase in the saliva can affect the detection of severe acute respiratory syndrome coronavirus 2 by real-time one-step polymerase chain reaction using saliva samples, Pathol. Res. Pract. 220 (2021) 153381.
[40] P. Pandit, J. Cooper-White, C. Punyadeera, High-yield RNA-extraction method for saliva, Clin. Chem. 59 (7) (2013) 1118–1122.
[41] Z. Khurshid, et al., Human saliva collection devices for proteomics: an update, Int. J. Mol. Sci. 17 (6) (2016).
[42] F. Michishige, et al., Effect of saliva collection method on the concentration of protein components in saliva, J. Med. Invest. 53 (1–2) (2006) 140–146.
[43] R. Sullivan, et al., An optimised saliva collection method to produce high-yield, high-quality RNA for translational research, PLoS One 15 (3) (2020) e0229791.
[44] H.Y. Ko, et al., Optimization and improvement of qPCR detection sensitivity of SARS-CoV-2 in saliva, Microbiol. Spectr. 11 (3) (2023) e0464022.
[45] ThermoFisher, Preserving RNA before Isolation. ThermoFisher.
[46] M. Navazesh, Methods for collecting saliva, Ann. N. Y. Acad. Sci. 694 (1993) 72–77.
[47] A. Shevchenko, et al., Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels, Anal. Chem. 68 (5) (1996) 850–858.
[48] B. Herbert, et al., Reduction and alkylation of proteins in preparation of two-dimensional map analysis: why, when, and how? Electrophoresis 22 (10) (2001) 2046–2057.
[49] L. Rochette, C. Vergely, Hans Selye and the stress response: 80 years after his "letter" to the Editor of Nature, Ann. Cardiol. Angeiol 66 (4) (2017) 181–183.

[50] A.J. Steckl, P. Ray, Stress biomarkers in biological fluids and their point-of-use detection, in: ACS Sensors, 2018, pp. 2025–2044.
[51] E. Balfoussia, et al., A proteomic study of plasma protein changes under extreme physical stress, J Proteomics 98 (2014) 1–14.
[52] A.M. Cooksey, et al., Identifying blood biomarkers and physiological processes that distinguish humans with superior performance under psychological stress, in: PLOS ONE, Public Library of Science, 2009, pp. 1–7.
[53] A. Zhang, et al., Salivary proteomics in biomedical research, Clin. Chim. Acta 415 (2013) 261–265.
[54] P. Zürbig, H. Jahn, Use of proteomic methods in the analysis of human body fluids in Alzheimer research, Electrophoresis 33 (24) (2012) 3617–3630.
[55] M. Naveed, et al., Plasma biomarkers: potent screeners of alzheimer's disease, Am J Alzheimers Dis Other Demen 34 (5) (2019) 290–301.
[56] K.E. Kaczor-Urbanowicz, et al., Saliva diagnostics - current views and directions, Exp Biol Med (Maywood) 242 (5) (2017) 459–472.
[57] J. Liu, et al., Saliva diagnostics: emerging techniques and biomarkers for salivaomics in cancer detection, Expert Rev. Mol. Diagn 22 (12) (2022) 1077–1097.
[58] T. Nonaka, D.T.W. Wong, Saliva diagnostics, Annu. Rev. Anal. Chem. 15 (1) (2022) 107–121.
[59] P. Ostheim, et al., Overcoming challenges in human saliva gene expression measurements, Sci. Rep. (2020) 11147.
[60] P.T.B. Ho, I.M. Clark, L.T.T. Le, MicroRNA-based diagnosis and therapy, Int. J. Mol. Sci. 23 (13) (2022).
[61] Y. Wu, et al., Circulating microRNAs: biomarkers of disease, Clin. Chim. Acta 516 (2021) 46–54.
[62] T.B. Bennike, et al., Comparing the proteome of snap frozen, RNAlater preserved, and formalin-fixed paraffin-embedded human tissue samples, EuPA Open Proteomics (2016) 9–18.
[63] W. Espinosa-de Aquino, et al., Protein and RNA extraction from mucosal swabs: a minimally invasive source of ecological data for studies of natural populations, in: Methods in Ecology and Evolution, John Wiley & Sons, Ltd., 2017, pp. 370–378.