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Proteomic Studies of Saliva: A Proposal for a Standardized Handling of Clinical Samples

François Chevalier · Christophe Hirtz · Sandrine Chay · Frédéric Cuisinier · Nicolas Sommerer · Michel Rossignol · Dominique Deville de Périère

Abstract

Background In recent years, differential analysis of proteins from human saliva, i.e., proteomic analysis, has received much attention mainly due to its unstressful sampling and its great potential for biomarker research. It is widely considered that saliva is a highly stable medium for proteins thanks to a large amount of antiprotease agents, even at ambient and physiological temperatures.

Objective To find the best protocol for the handling of samples, we have investigated the stability of saliva proteins stored at different temperatures (from −80 to 20°C) by one- and two-dimensional electrophoresis.

Results At 20°C, no major changes were observed on protein one-dimensional profiles following 1 day of storage; however, between 7 days and 30 days, the native alpha-amylase band decreased slightly to give several bands with molecular weight between 35 and 25 kDa. The same phenomenon appeared after 30 days of storage at 4°C. Two-dimensional analysis of salivary maps revealed degradation from day 7 of several protein groups for samples stored at 20°C.

Conclusion All these findings have to be carefully considered when saliva is collected for clinical proteomic analysis. We can conclude that, to maintain the optimum stability of saliva proteins, saliva samples should be collected on ice followed by the addition of protease inhibitor cocktail, centrifuged to remove insoluble material, and stored at −20 or −80°C.

Keywords Saliva · Stability · Storage · Protocol · Proteomic

Introduction

Saliva has become popular in recent decades as a medium for the measurement of numerous biomolecules [1–4]. The greatest advantage, when compared to blood sample collection, is that saliva is readily accessible and collectible. Consequently, it can be used in clinically difficult situations, such as in children, handicapped, and anxious patients, where blood sampling could be a difficult act to perform [5].

A wide range of biomolecules are now monitored in saliva [6, 7]. Proteins and peptides were extensively studied in saliva for biomarker research; these include the quanti-
fication of steroid hormones such as cortisol or progesterone [8–11], and the detection of various therapeutic or illicit drugs [12]. Tumor markers that were identified in saliva have been used to screen malignant diseases [13–15]. Antibodies against viruses and viral components can also be detected in saliva, therefore making it easier to diagnose various infections such as, for example, hepatitis A, B, or C; HIV; rubella; and dengue [16–19]. Identification of all saliva proteins [20–39] allowed comparative analysis of proteomic maps of healthy and ill people, which revealed differentially expressed proteins as potential biomarkers [26, 28, 32, 35, 36, 38].

On a recent summary about the proteomic technologies currently used, Hu et al. pointed out the critical challenges and perspectives for this emerging field [40]. The first point was the need to standardize the sample collection, preparation, and handling procedures. Indeed, to ensure comparable analyses of saliva and, thus, results between studies and labs, careful attention to methodological detail is required. Collection, storage conditions, and processing of saliva samples are key steps to guarantee reproducibility and validity of measurements [40–42].

In addition to salivary gland secretions (water, proteins, enzymes, electrolytes, and small organic molecules), blood derivatives from gingival crevicular fluid or oral wounds, bacteria, fungi, desquamated epithelial cells, expectorated bronchial and nasal secretions, or food debris have also been found in saliva [4]. Many of these components may have damaged molecules of interests for proteomic analysis. Careful control of temperature during saliva collection and salivary sample storage is crucial; additionally, most insoluble materials can be eliminated by centrifugation or filtration. The use of a protease inhibitor cocktail can help to reduce protein degradation during saliva sample collection and processing. A review of previous protocols used for proteomic analysis of saliva showed variations in several parameters such as temperature at sample collection, the addition of a protease inhibitor cocktail, the removal of insoluble material and the temperature of storage of saliva samples (Table 1). This can be explained by the fact that, for many decades, it was largely believed that saliva contains a high amount of protease inhibitors, which confers a great stability on proteins.

Saliva storage protocols have been extensively analyzed for the stability of steroids [43], progesterone [44, 45], immunoglobulin A and lysozyme [46], RNA of hepatitis C virus, and blood group antigen detection [47, 48], but never for a proteome analysis, with the exception of Schipper et al. [39], who recently showed differences on surface enhanced laser desorption/ionization-time-of-flight (TOF) mass spectra of saliva samples as a function of storage temperature, centrifugation speed, and the use of protease inhibitors.

In the present paper, we propose to study the stability of the major salivary proteins stored at various storage temperatures (−80, −20, 4, and 20°C) by one-dimensional and bidimensional electrophoresis to estimate the rate of stability of saliva proteins and, in the case of degradation, which proteins or group of proteins were the most susceptible. The aim of this work is to establish a robust and standardized protocol for collection and storage of saliva that could be widely used for proteomic analysis of human saliva.

Materials and Methods

Collection of Saliva

Whole saliva was collected from a healthy subject 2 h after breakfast time. Before sample collection, the subjects brushed their teeth and rinsed their mouth with water. To help saliva production, individuals drank a glass of water 15 min before collection, and then, stimulated saliva was collected on ice for 5 min by chewing on paraffin wax and spitting intermittently. Saliva samples were centrifuged at 15,000×g for 15 min at 4°C to eliminate insoluble material. A sample was kept with insoluble material (without the centrifugation step) to investigate the influence of bacteria, mucine, and other insoluble material on protein stability.

Storage Kinetics of Saliva

Clear saliva was aliquoted (1 ml) into microcentrifuge tubes and stored at either 4°C or ambient temperature. At regular intervals (10 min, 1 h, 1 day, 7 days, 30 days), samples were placed in a −20°C freezer until further analysis by mono- and two-dimensional electrophoresis. A sample with protease inhibitor mixture (Complete®, Roche, Penzberg, Germany) was also prepared and placed at ambient temperature for 30 days. Control samples were also stored at either −80 or −20°C for 30 days.

Extraction of Salivary Protein

Proteins were first extracted using the trichloroacetic acid (TCA)/acetone method [49]. Briefly, proteins were precipitated using 90% acetone (v/v), 10% TCA (v/v), and 0.07% 2-mercaptoethanol (v/v). After incubation overnight at −20°C, insoluble material was centrifuged at 42,000×g for 10 min at 4°C. Pellets were washed three times with pure acetone containing 2-mercaptoethanol, air dried, and solubilized in 9 M urea, 4% 3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid (w/v), 0.05% Triton X100 (v/v), and 65 mM dithiothreitol (DTT). Protein content was estimated using the Bradford method [50].
One-Dimension Electrophoresis

Saliva samples (50 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 11% acrylamide gels (1.5 mm thick) according to Laemmli [51]. Gels were stained with the silver staining procedure of Vorum [52].

Two-Dimensional Gel Electrophoresis

Precast 18 cm IPG strips pH 4–7 (Amersham Biosciences, Orsay, France) were rehydrated directly overnight with protein samples (100 μg). Isoelectric focusing was carried out using the IPGphor™ isoelectric focusing system for a total of ca 70,000 V.h. Thereafter, strips were equilibrated for 15 min in 6 M urea, 50 mM Tris HCl buffer at pH 8.8, 30% glycerol (v/v), 2% SDS (w/v), and 65 mM DTT, and then for 15 min in the same solution with 65 mM iodoacetamide instead of DTT. Proteins were finally separated on 12% SDS-polyacrylamide gels, at constant voltage (150 V) and 10°C, using an Iso-DALT electrophoresis unit (Amersham Biosciences). Gels were stained with colloidal Coomassie blue [53]. Gel images were digitalized at 300 dpi with a GS 710 densitometer (Biorad, Hercules, CA, USA) and analyzed using the Progenesis software (Non-linear Dynamics, Newcastle upon Tyne, UK).

### Table 1  A review of previous processing protocols used for proteomic analysis of saliva

| Ref. | Saliva type                        | Temperature of collection | Addition of protease inhibitor | Removal of unsoluble material | Temperature of samples storage | Proteomic analysis                                                                 |
|------|-----------------------------------|---------------------------|--------------------------------|-------------------------------|-------------------------------|----------------------------------------------------------------------------------|
| 54   | Stimulated parotid saliva         | Ice                       | No                             | No                            | −20°C                         | HPLC and N-terminal sequencing                                                      |
| 20   | Whole saliva                      | Unknown                   | No                             | No                            | −20°C                         | SDS-PAGE and MALDI-TOF                                                            |
| 21   | Stimulated glandular and whole saliva | Ice                 | No                             | No Centrifugation             | −20°C                         | 2DE and MALDI-TOF                                                                |
| 22   | Stimulated whole saliva           | Ice                       | No                             | Centrifugation                 | No storage: direct analysis     | 2DE and MALDI-TOF                                                                |
| 23   | Unstimulated whole saliva         | Unknown                   | No                             | Centrifugation                 | −20°C                         | 2DE and MALDI-TOF, Q-TOF, N-terminal sequencing and Western blotting               |
| 25   | Whole saliva                      | Ice                       | Yes                            | No                            | −20°C                         | 2DE and MALDI-TOF, Q-TOF, N-terminal sequencing and Western blotting               |
| 27   | Stimulated parotid saliva         | Ice                       | No                             | No Centrifugation             | −20°C                         | 2DE and MALDI-TOF, Q-TOF, N-terminal sequencing and Western blotting               |
| 29   | Whole saliva                      | Ice                       | Yes                            | No                            | −80°C                         | Shotgun proteomics and 2DE and MALDI-TOF                                          |
| 30   | Stimulated whole saliva           | Ice                       | Yes                            | No                            | −80°C                         | 2DE and MALDI-TOF                                                                |
| 31   | Stimulated whole saliva           | Ice                       | Yes                            | No                            | −80°C                         | 2DE and MALDI-TOF                                                                |
| 32   | Stimulated whole saliva           | Ice                       | Yes                            | No                            | −80°C                         | 2DE and MALDI-TOF                                                                |
| 33   | Unstimulated whole saliva         | Unknown                   | No                             | No                            | −80°C                         | 2DE and MALDI-TOF                                                                |
| 34   | Unstimulated whole saliva         | Unknown                   | No                             | No                            | −80°C                         | 2DE and MALDI-TOF                                                                |
| 35   | Parotid saliva                    | Ice                       | No                             | No                            | −80°C                         | 2D-DIGE and SELDI-TOF                                                            |
| 37   | Unstimulated whole saliva, parotid| Ice                       | No                             | No                            | −80°C                         | 2DE and MALDI-TOF                                                                |
| 38   | Unstimulated whole saliva         | Ice                       | No                             | No                            | −80°C                         | 2DE and MALDI-TOF                                                                |
| 39   | Stimulated and unstimulated whole saliva | Unknown               | Yes                            | No                            | −80°C                         | SELDI-TOF                                                                        |

MS = mass spectrometry, SELDI = surface enhanced laser desorption/ionization
Image Analysis

Gel images were analyzed in triplicate with the Progenesis Workstation software v.2003.2 (Perkin Elmer Life Sciences, Cambridge, UK). An automatic averaged gel experiment was performed with single analytic gels using the control sample gels as reference. For each sampling time, eight areas were selected and compared. Images were first warped to the reference gel and areas were then matched. A combined algorithm warping and matching was used. The “progenesis background” was used as the background method and the spot volume was determined as percentage of total volume of all spots on respective gels.

Matrix-Assisted Laser Desorption/Ionization-TOF Mass Spectrometry Analysis

Spots were excised from gels after two-dimensional electrophoresis by hand and processed using a Packard Multiprobe II liquid handling robot (Perkin Elmer, Courtaboeuf, France). After washing with water, 25 mM ammonium bicarbonate, acetonitrile/25 mM ammonium bicarbonate (1:1, v/v) and acetonitrile, gel fragments were dried at 37°C. Protein digestion was carried out at 37°C for 5 h after addition of 0.125 μg trypsin. Resulting fragments were extracted twice with 50 μL of acetonitrile/water (1:1, v/v) containing 0.1% trifluoroacetic acid for 15 min. Pooled supernatants were concentrated to a final volume of ca 20 μL. Peptides were desalted and concentrated to a final volume of 3 μL with C18 Zip-Tip microcolumns and immediately spotted onto the matrix-assisted laser desorption/ionization (MALDI) target by the robot. Mass spectra were recorded in the reflector mode on a BiFlex III MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Automatic annotation of monoisotopic masses was performed using Bruker’s SNAPTM procedure. The MASCOT search engine software (Matrix Science, London, UK) was used to search the NCBInr database. The following parameters were used: mass tolerance of 100 ppm, a minimum of five peptides matching to the protein, carbamidomethylation of cysteine as fixed modification, oxidation of methionine, and pyroglutamylation of glutamine as variable modifications and one missed cleavage allowed.

Results

Collection and Storage of Saliva Samples

Stimulated saliva was collected between 9 a.m. and 10 a.m. A male volunteer (age, 33 years) was asked to refrain from eating, drinking, smoking, or oral hygiene procedures, and a water mouth-rinse was required prior to sample collection. Stimulated saliva was collected on ice and clarified by centrifugation to eliminate mucine, cell debris, and insoluble material. A control sample was kept without centrifugation (unclear saliva). Samples (1 ml) of clear saliva were aliquoted into 1.5-ml microtubes and incubated at either −80, −20, 4, and 20°C. A control sample (0 min) was frozen at −20°C immediately after collection. The kinetics of storage was studied with samples kept at both 4 and 20°C for 10 min, 1 h, 1 day, 7 days, and 30 days. A cocktail of protease inhibitor was added to a sample maintained at both 4 and 20°C for 30 days.

One-Dimensional Electrophoresis Analysis of Stored Saliva

The same volume of clear or unclear saliva samples was separated by SDS-PAGE (Fig. 1). According to such one-dimensional electrophoresis, only profile changes of proteins of similar molecular weights could be observed.

A large diversity of molecular weights was observed with the initial sample frozen immediately after collection (Fig. 1a, lane 1). Major bands were observed close to 50 and 15 kDa, corresponding to native alpha-amylase and cystatins, respectively. Minor and diffuse bands were observed between 50 and 20 kDa, corresponding to an abundant variety of salivary proteins. According to the storage kinetics, no major changes were observed on protein profiles of samples stored for 60 min at ambient temperature (Fig. 1a, lane 3). At 1 day, the band intensity just below the native alpha-amylase seemed to be less intense. At 7 days, the intensity of the native alpha-amylase band (57 kDa) decreased slightly, resulting in several bands between 35 and 25 kDa. This phenomenon seemed to be enhanced after 30 days storage at ambient temperature (Fig. 1a, lane 6). The addition of protease inhibitors was not able to block such degradation; no real difference was observed at 30 days between samples containing cocktail inhibitors or not.

In contrast, when saliva was stored at 4°C, no such degradation was observed until 7 days (Fig. 1b, lane 4). From days 7 to 30, the protein profile seemed to be slightly less intense in the molecular weight range between 50 and 25 kDa, but without any band appearance. The addition of protease inhibitors successfully blocked such degradation at day 30 (Fig. 1b, lane 6). In contrast, when insoluble material was not removed, saliva proteins were completely disappeared at either 4 or 20°C (Fig. 1a, lane 8, and Fig. 1b, lane 7, respectively). In this case, proteins were certainly degraded by proteases from bacteria and cells of the oral cavity. The storage of saliva samples for 30 days at −20 and −80°C allowed a complete preservation of saliva proteins, lanes 8 and 9, respectively (Fig. 1b).
Two-Dimensional Electrophoresis Analysis of Stored Saliva

To obtain more detailed information, two-dimensional electrophoresis was used to analyze changes in specific protein groups. Based on the results of one-dimensional analysis, the times at which protein degradation was most affected were selected for the two-dimensional electrophoresis separation: in addition to control samples (0 day and 30 days with protease inhibitors), we selected samples stored at 20°C for 1, 7, and 30 days to be analyzed. The 30 major protein spots were selected on two-dimensional gels (Fig. 2) and identified by peptide mass fingerprinting (Table 2). To compare protein amount during storage times, we decided to group proteins from the same family and/or gel area (Fig. 2 and Table 2). All the areas were calculated as a mean of three replicates of the same extract and were matched together to analyze the evolution of relative intensity according to the storage time (Fig. 3). Areas A to D were strongly affected by storage time at 20°C. Area A, corresponding to serum albumin, disappeared on the bidimensional map from 1 day and only half of the spot intensity was maintained at 30 days when a protease inhibitor cocktail was added to the sample. In the case of area B, the quantity of salivary acidic prolin rich protein (PRP) decreased dramatically up to 7 days at ambient temperature, with only a small amount of the corresponding spots still observable at days 7 and 30. The same result was obtained with spots of area C, corresponding to prolactin inductive proteins and salivary acidic protein-1. Spot identified as salivary cystatins SA-1 (area D) had disappeared totally by day 7, showing its low stability at 20°C. The addition of a protease inhibitor cocktail did not affect the degradation of these proteins after 30 days storage at ambient temperature. The protease inhibitor cocktail tablets used (Complete Mini®, Roche) were designed to inhibit mainly serine-, cysteine- and metallo-proteases. Nevertheless, the relatively quick degradation of proteins could be attributed to residual protease activity or to the presence of acidic proteases such as aspartic protease.
Table 2  Identification of the 30 most abundant spots from the two-dimensional gel of whole saliva (see Fig. 2), by peptide mass fingerprint MALDI-TOF

| No | Group | Protein name                  | Ref. | % Cov. | MW (kDa) theoretical | Observed | pI theoretical | Observed |
|----|-------|-------------------------------|------|--------|----------------------|----------|----------------|----------|
| 1  | A     | Serum albumin                 | P02768 | 29     | 66.5                 | 68       | 5.67           | 5.5      |
| 2  | A     | Serum albumin                 | P02768 | 25     | 66.5                 | 68       | 5.67           | 5.6      |
| 3  | A     | Serum albumin                 | P02768 | 25     | 66.5                 | 68       | 5.67           | 5.8      |
| 4  | A     | Serum albumin                 | P02768 | 27     | 66.5                 | 68       | 5.67           | 6.0      |
| 5  | A     | Serum albumin                 | P02768 | 25     | 66.5                 | 68       | 5.67           | 6.2      |
| 6  | F     | Salivary-amylase              | P04745 | 42     | 55.9                 | 59       | 6.34           | 5.5      |
| 7  | F     | Salivary-amylase              | P04745 | 33     | 55.9                 | 59       | 6.34           | 5.8      |
| 8  | F     | Salivary-amylase              | P04745 | 28     | 55.9                 | 59       | 6.34           | 6.0      |
| 9  | F     | Salivary-amylase              | P04745 | 51     | 55.9                 | 59       | 6.34           | 6.1      |
| 10 | F     | Salivary-amylase              | P04745 | 50     | 55.9                 | 59       | 6.34           | 6.3      |
| 11 | F     | Salivary-amylase              | P04745 | 51     | 55.9                 | 59       | 6.34           | 6.5      |
| 12 | F     | Salivary-amylase              | P04745 | 50     | 55.9                 | 59       | 6.34           | 6.7      |
| 13 | G     | Zinc-alpha-2-glycoprotein     | P25311 | 52     | 32.1                 | 42       | 5.58           | 5.1      |
| 14 | G     | Zinc-alpha-2-glycoprotein     | P25311 | 53     | 32.1                 | 42       | 5.58           | 5.3      |
| 15 | G     | Zinc-alpha-2-glycoprotein     | P25311 | 57     | 32.1                 | 42       | 5.58           | 5.5      |
| 16 | G     | Zinc-alpha-2-glycoprotein     | P25311 | 53     | 32.1                 | 42       | 5.58           | 5.7      |
| 17 | B     | Salivary acidic PRP           | P02810 | 36     | 15.4                 | 26       | 4.63           | 4.1      |
| 18 | B     | Salivary acidic PRP           | P02810 | 42     | 15.4                 | 25       | 4.63           | 4.2      |
| 19 | C     | Prolactin inducible           | P12273 | 38     | 13.5                 | 18       | 5.47           | 4.2      |
| 20 | C     | Prolactin inducible           | P12273 | 50     | 13.5                 | 18       | 5.47           | 4.5      |
| 21 | C     | Prolactin inducible           | P12273 | 43     | 13.5                 | 18       | 5.47           | 5.0      |
| 22 | C     | Salivary acidic protein 1     | P01036 | 27     | 14.2                 | 16       | 4.83           | 4.2      |
| 23 | C     | Salivary acidic protein 1     | P01036 | 29     | 14.2                 | 16       | 4.83           | 4.4      |
| 24 | C     | Salivary acidic protein 1     | P01036 | 34     | 14.2                 | 16       | 4.83           | 4.7      |
| 25 | D     | Salivary cystatin SA-1        | P01037 | 40     | 14.3                 | 16       | 6.92           | 7.0      |
| 26 | E     | Salivary-amylase              | P04745 | 34     | 55.9                 | 30       | 6.34           | 6.4      |
| 27 | E     | Salivary-amylase              | P04745 | 22     | 55.9                 | 35       | 6.34           | 6.5      |
| 28 | E     | Salivary-amylase              | P04745 | 35     | 55.9                 | 30       | 6.34           | 6.7      |
| 29 | E     | Salivary-amylase              | P04745 | 43     | 55.9                 | 28       | 6.34           | 6.8      |
| 30 | E     | Salivary-amylase              | P04745 | 27     | 55.9                 | 27       | 6.34           | 7.1      |

Protein reference (Ref.) corresponds to the Swiss-Prot/NCBI accession number; sequence coverage (% cov.) is given as percentage.

Fig. 3  Total protein amount of spot groups from the two-dimensional separation (i.e., see Fig. 2) as a function of storage time at ambient temperature. a–g groups a–g.
In contrast, the opposite result was obtained with areas E, F, and G, in which no real degradation was observed. The corresponding proteins, alpha-amylase (groups E and F) and zinc-alpha-2 glycoprotein (group G), seemed to resist degradation up to 30 days storage at ambient temperature. Nevertheless, after 1 day of storage, a 15% decrease of area F spot intensities was observed with a concomitant increase of area E spots. It could be hypothesized that native alpha-amylase was degraded to constitute a part of alternative alpha-amylase forms. These lower-molecular-weight forms of alpha-amylase were previously described as a complex but stable protein pattern [31]. Certainly a part of these alternative alpha-amylase spots remained from the degradation of the native alpha-amylase form [3, 4], but another part, which constitutes the stable pattern [31], seemed not to be affected by degradation. The example of these alternative alpha-amylase spots clearly demonstrated the importance of a strong control of protease actions to understand and compare proteomes.

Concluding Remarks

As saliva is now frequently used as a diagnostic biological fluid, our work demonstrated the necessity for a well-controlled storage protocol of saliva samples to ensure quantitative and reproducible clinical analysis. Of course, storage temperatures such as −20 and −80°C are now commonly used to store biospecimens, but such storage conditions could be considered as excessive owing to the fact that saliva contains a high amount of protease inhibitors which can prevent protein degradation.

Our results clearly showed the weak stability of several salivary proteins. According to one- and two-dimensional electrophoresis studies (Table 3), the stability of clear saliva was preserved until 1 day at 20°C and 7 days at 4°C. After 30 days at 20°C, almost half of the major salivary proteins were degraded even if a protease inhibitor cocktail was added to the sample.

According to these results, and in agreement with Schipper et al. [39], the protocol used by Hu et al. [29] seemed to be the best to maintain the optimum stability of salivary proteins. In case of a clinical comparison with a pathological condition, control saliva samples should be collected from a healthy nonsmoking subject, in the morning, at least 2 h after eating, and the mouth should be rinsed with water. After collection, saliva samples should be stored in a freezer at −20°C, and during sampling, saliva should be kept on ice with a protease inhibitor cocktail and centrifuged to remove insoluble material and then stored at −80°C.

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Table 3 Stability of saliva proteins according to the one-dimension and two-dimensional electrophoresis experiments

| Temperature of samples storage | Duration of storage | Addition of protease inhibitor | Removal of unsoluble material | Degradation of proteinsa | Protein groups sensibleb |
|-------------------------------|---------------------|-------------------------------|------------------------------|---------------------------|--------------------------|
| 20°C                          | 0                   | –                             | +                           | –                         | None                     |
| 20°C                          | 10 min              | –                             | +                           | –                         | A, B, C, D, G            |
| 20°C                          | 60 min              | –                             | +                           | –                         | A, B, C, D, G            |
| 20°C                          | 1 day               | –                             | +                           | –                         | A                        |
| 20°C                          | 7 days              | –                             | +                           | +                         | A, B, C, D, G            |
| 20°C                          | 30 days             | –                             | +                           | +                         | A, B, C, D, G            |
| 20°C                          | 30 days             | +                             | +                           | +                         | A, B, C, D, G            |
| 20°C                          | 30 days             | –                             | +                           | –                         | A                        |
| 4°C                           | 10 min              | –                             | +                           | –                         | /                        |
| 4°C                           | 60 min              | –                             | +                           | –                         | /                        |
| 4°C                           | 1 day               | –                             | +                           | –                         | /                        |
| 4°C                           | 7 days              | –                             | +                           | +                         | /                        |
| 4°C                           | 30 days             | –                             | +                           | –                         | /                        |
| 4°C                           | 30 days             | +                             | +                           | –                         | /                        |
| −20°C                         | 30 days             | –                             | +                           | –                         | /                        |
| −80°C                         | 30 days             | –                             | +                           | –                         | /                        |

a One-dimensional electrophoresis experiment
b Two-dimensional electrophoresis experiment
41. Nimmagudda RR, Ramanathan R, Putcha L. A method for preserving saliva samples at ambient temperatures. Biochem Arch 1997;13:171–8.
42. Landi MT, Caporaso N. Sample collection, processing and storage. IARC Sci Publ 1997;142:223–36.
43. Groschl M, Wagner R, Rauh M, Dorr HG. Stability of salivary steroids: the influences of storage, food and dental care. Steroids 2001;66:737–41.
44. Banerjee S, Levitz M. On the processing of saliva samples for progesterone assay. Steroids 1983;42:539–47.
45. Banerjee S, Levitz M, Rosenberg CR. On the stability of salivary progesterone under various conditions of storage. Steroids 1985;46:967–74.
46. Ng V, Koh D, Fu Q, Chia SE. Effects of storage time on stability of salivary immunoglobulin A and lysozyme. Clin Chim Acta 2003;338:131–4.
47. Roy KM, Bagg J, McCarron B. The effect of saliva specimen collection, handling and storage protocols on hepatitis C virus (HCV) RNA detection by PCR. Oral Dis 1999;5:123–7.
48. Kim W, Kim YK, Chung SC, Lee SW, Kho HS. Detection of ABH blood group antigens in the saliva of Koreans and their stability according to storage of saliva samples. Forensic Sci Int 2002;129:58–63.
49. Chevalier F, Martin O, Rofidal V, Devauchelle AD, Rossignol M. Proteomic investigation of natural variation between Arabidopsis ecotypes. Proteomics 2004;4:1372–81.
50. Bradford MA. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
51. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.
52. Mortz E, Krogh TN, Vorum H, Gorg A. Improved silver staining protocols for high sensitivity protein identification using matrix-assisted laser desorption/ionization-time of flight analysis. Proteomics 2001;1:1359–63.
53. Chevalier F, Rofidal V, Vanova P, Bergoin A, Rossignol M. Proteomic capacity of recent fluorescent dyes for protein staining. Phytochem 2004;65:1499–506.
54. Perinpanayagam HE, Van Wuyckhuyse BC, Ji ZS, Tabak LA. Characterization of low-molecular-weight peptides in human parotid saliva. J Dent Res 1995;74:345–50.
55. Wilmarth PA, Riviere MA, Rustvold DL, Lauten JD, Madden TE, David LL. Two-dimensional liquid chromatography study of the human whole saliva proteome. J Proteome Res 2004;3:1017–23.