MATERIALS AND METHODS
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3.1 Collection of Samples

The saliva of women was collected as per the spitting method (Navazesh, 1993; Bosch et al., 1996) and the sample was processed in preweighed ice-chilled tubes and the collection period was about 10 min. The saliva was collected from 150 different women volunteers (20-30 years) during various periods viz., preovulatory (6-12 days), ovulatory (13-14 days), postovulatory phases (15-26 days) and also from prepubertal (7-9 years) and menopause stages (above 45 years). The volunteers were instructed to abstain from smoking and drinking 10 hrs prior to the sample collection. The volunteers were also asked for brushing the tooth to prevent minimal gingival bleeding.

For the present study, the volunteers from women's hostel, Bharathidasan University as well as town and rural areas of Tiruchirappalli were clinically observed and tested. The saliva sample was categorized into three phases, viz., Preovulatory, Ovulatory and Postovulatory phases. The volunteers were carefully cautioned not to use any contraceptives pills or natural medicines during the course of study. The samples were collected in a sterile vial during morning hours and brought to the laboratory for microscopic examination.

3.2.1 SALIVARY FERNING METHOD

At least 5 cycles were observed to confirm the length of period from the volunteers prior to sample collection. Those women exhibited normal 28 days cycle (28 ± 4 days cycle length consecutively) were taken as volunteers and the saliva sample was collected. The fern pattern was analyzed using glass slide, by
To Collect the Sample: (Human Saliva)

1) Remove the cotton roll from the enclosed collection tube.
2) Place the cotton roll in your mouth and lightly chew on it until the cotton is wet (about 2 or 3 minutes).
3) Place the wet roll back in the collection tube and replace the cap.
4) Insert the sealed collection tube into the crush-proof mailing container.
5) Place the saliva sample in your home freezer until it is frozen solid.
6) Once the sample is frozen, place the collection container and store at -70 °C until analysis.
adding one drop of saliva, which was smeared by other slide; the fern like crystals was formed in each stage of menstrual cycle.

### 3.2.2 ULTRASOUND FINDINGS

An examination was performed early in the cycle and daily, from day 8 or day 9, over the period of expected ovulation and into the midluteal phase. If the subject had a long cycle, the ovaries were examined less regularly until a small follicle was visualized, and then daily during its development. Each ovary was scanned in the transverse and longitudinal planes and photographs were taken. The follicles were measured in these planes at their greatest diameter by reference to a calibrated centimeter scale incorporated into the photograph. The volume of a follicle was calculated with the formulae $4/3\pi r^3$ (where $r$ is the radius) if the diameters did not differ by >0.5mm. If the follicle was ovoid, the volume was calculated from the formula $4/3\pi r_1 r_2 r_3$, as described by Rainefenning et al., (2004), where $r_1$, $r_2$ and $r_3$ represent the radii of the three largest diameters.

### 3.2.3 SALIVARY HORMONE PROFILES

**RADIOIMMUNOASSAY FOR SALIVARY HORMONES IN WOMEN SALIVA DURING MENSTRUAL CYCLE**

1. **Salivary Luteinising hormone (LH) assay (Knobil, 1980)**

   **Mouse Monoclonal anti-a-LH antibody coated micro titer plate with 96 wells.**
   
   i) Enzyme Conjugate Reagent, 13 ml.
   
   ii) LH reference standards, containing 0, 5, 15, 50, 100 and 200 mIU/ml. (WHO, 1st IRP, 68/40), Lyophilized.
   
   iii) TMB Reagent (One-Step), 11 ml Protect from Light.
   
   iv) Stop Solution (1N HCl), 11 ml.
2. **Salivary follicle stimulating hormone** (FSH) assay (Marshall, 1975)

**Mouse Monoclonal anti-a-FSH antibody coated micro titer plate with 96 wells.**

i. Enzyme Conjugate Reagent, 13 ml.

ii. FSH reference standards, containing 0, 5, 15, 50, 100, and 200 mIU/ml (WHO, 2nd IRP 78/549) human FSH, lyophilized, 1 set.

iii. TMB Reagent (One-Step), 11 ml. Protect from Light.

iv. Stop Solution (1N HCl), 11 ml.

**REAGENT PREPARATION**

1. All reagents were allowed to reach room temperature (18-25°C) before use.

2. Each was reconstituted at lyophilized standard with 1.0 ml distilled water and allowed the reconstituted material to stand for at least 20 min and mixed gently. Reconstituted standards would be stable for up to 30 days when the seal stored at 2-8°C.

**ASSAY PROCEDURE**

1. Secured the desired number of coated wells in the holder.

2. Dispensed 50 µl of standard, specimens, and controls into appropriate wells.

3. Dispensed 100 µl of Enzyme Conjugate Reagent into each well.

4. Gently mixed for 30 seconds. It is very import to have complete mixing, used a micro plate rotator.

5. Incubated at room temperature (18-25°C) for 45 min.

6. Removed the incubation mixture by flicking plate contents into sink.

7. Rinsed and flick the micro titer wells 5 times with distilled or deionized water.
8. Shake the wells sharply onto absorbent paper or paper towels to remove all residual water droplets.
9. Dispensed 100 μl TMB Reagent into each well. Gently mixed for 5 sec.
10. Incubated at room temperature in the dark for 20 min.
11. Stopped the reaction by adding 100 μl of stop solution to each well.
12. Gently mixed for 30 sec. It is important to make sure that all the blue color changes to yellow color completely.
13. Read the optical density at 450 nm with a micro titer plate reader within 15 min.

**CALCULATION OF RESULTS**

1. Calculated the average absorbance values (A450) for each set of reference standards, control, and samples.
2. Constructed using a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in mIU/ml on linear graph paper, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis.
3. Using the mean absorbance value for each sample, the corresponding concentration of LH and FSH in mIU/ml from the standard curve was determined.
4. The cross reactivity of this antiserum were 0.26%, 1%, non-detectable and 0.001% respectively. The intra and inter assay coefficients of variation were 0.12-1.0 mIU/L for LH and 1.0-2.0 mIU/L for FSH respectively.

3. **SALIVARY ESTRADIOL DETERMINATION** (Worthman et al., 1990)

Estradiol was estimated in women saliva samples using solid phase (antibody coated tubes) RIA system where in the antibody was immobilized on the wall of tubes. This method was based on
the competition of unlabelled estradiol in the standard or samples and estradiol [125i] for the binding sites on the antibody coated tubes. They were allowed to reach equilibrium and at the end of incubation period, the antibody bound and decanting the tubes separates free antigens. Estradiol concentration of samples was quantified by measuring the radioactivity associated with bound fraction of samples and standards.

**Reagents**

Buffered [125i] estradiol: 1 vial of iodinated estradiol with a specific activity of 3 microcuries or 111 kilobecquerels was used.

Estradiol calibrators: Estradiol standards of 0, 20, 50, 150, 500, 1800 and 3600 pg/ml were used as calibrators to plot the standard graph for reference.

**Procedure**

The assay was carried out in duplicate with total, non-specific binding, maximum binding, standards, quality controls and serum sample tubes.

i. Plain 12 X 75 mm polypropylene tubes in duplicate were used for total and non-specific binding.

ii. Estradiol antibody – coated polypropylene tubes were used for maximum binding, standard and serum samples.

**Reagents were added as follows:**

1. Total count tube
   
   1 ml of buffered [125i] estradiol

2. Non-specific binding tubes (NSB)
   
   1 ml of buffered [125i] estradiol and 100 μl of zero calibrator (0 ng/ml) (Uncoated plain tubes)
3. Maximum binding tubes
   1 ml of buffered [125i] estradiol Antibody-coated tubes

4. Standard tubes
   100 µl of different concentration of standards (20-3600 pg/ml) and 1 ml of buffered [125i] estradiol.

5. Unknown (saliva) tubes
   100 µl of saliva sample and 1ml of buffered [125i] estradiol.

Tubes were agitated and vortexed gently. They were incubated for 3 hrs at room temperature. At the end of incubation, all tubes were thoroughly decanted (except total count tubes) and counted for 1 min in a microprocessor based LKB rack gamma counter. Estradiol concentration in the samples were calculated from a logit-log representation of the calibration curve and expressed as pg/ml.

The sensitivity of the assay was 5 pg/ml using the 3 hr room temperature procedure. The maximum binding was 35-45%. The antiserum was highly specific for estradiol with very low cross reactivity to other compounds, which might be present in the sample. The cross reactivity of this antiserum were 0.32%, 1%, non-detectable and 0.001% respectively. The intra and inter assay coefficients of variation were 6.2 – 8.4% and 9.7% respectively.

4. SALIVARY PROGESTERONE (Finn et al., 1998)

Saliva progesterone was also estimated using solid phase (antibody coated tubes) RIA procedure. The principle was same as described for estradiol and mentioned elsewhere in the thesis.
Reagents

1. Buffered [125i] progesterone
   1 vial of iodinated progesterone with a specific activity of 4.5 microcuries or 167 kilobequerels units was provided.

2. Progesterone calibrators
   Progesterone standards of 0, 0.1, 0.5, 2.0, 10.0, 20.0 and 40.0 ng/ml were used as calibrators to plot the standard graph for reference.

Procedure

The assay was carried out in duplicate with total, non-specific, maximum binding, quality control, standard and saliva samples. The procedure was same as described for estradiol assay. Saliva progesterone was calculated from a logit-log representation of the calibration curve and expressed as ng/ml. The sensitivity of the assay was 0.01% ng/ml. The maximum binding was 40%. The antiserum was highly specific for progesterone with very low cross reactivity to other compounds such as cortisone, 0.4%, 17α-hydroxy-progesterone – 0.3% and testosterone non-detectable present in the saliva. The intra and interassay coefficients of variation were 5.0 – 9.0% and 7.9% respectively.

5. SALIVARY ESTRONE-1-GLUCURONIDE (E-1-G) (Folan et al., 1989)

REAGENTS Required

1. Rabbit Anti-Estrone Antibody Coated Microwell Plate-Break Apart Wells - Ready To Use.
   Contents: One 96 well (12x8) polyclonal antibody-coated microwell plate in a resealable pouch with desiccant.
   Storage: Refrigerate at 2-8°C
2. Estrone-Biotin Conjugate Concentrate - Requires Preparation.
Contents : Estrone-biotin conjugates in a protein-based buffer with a non-mercury preservative.
Volume : 200 µl/vial
Storage : Refrigerate at 2-8°C
Stability : 12 months or as indicated on label.
Preparation : See No.4 below.

Contents : Avidin-HRP conjugates in a protein-based buffer with a non-mercury preservative.
Volume : 200 µl/vial
Storage : Refrigerate at 2-8°C
Stability : 12 months or as indicated on label.
Preparation : See No. 4 below.

4. Preparation of Estrone-Biotin and Avidin-HRP Conjugate
Diluted both the estrone-biotin and avidin-HRP concentrates 1:100 into the same solution of assay buffer and mix thoroughly (e.g. To a tube containing 2 ml of assay buffer add 20 µl of estrone-biotin and 20 ml of avidin-HRP concentrates).

5. Estrone Calibrators - Ready To Use.
Contents : Six vials containing estrone in a protein-based buffer with a non-mercury preservative. Prepared the buffer by spiking with a defined quantity of estrone.
Storage : Refrigerate at 2-8°C
Stability : 12 months in unopened vials or as indicated on label.
Once opened, the standards should be used within
14 days or aliquoted and stored frozen. Avoid multiple freezing and thawing cycles.

6. Control - Ready To Use.
Contents : One vial containing estrone in a protein-based buffer with a non-mercury preservative. Prepared the buffer by spiking with a defined quantity of estrone. Refer to vial label for expected value and acceptable range.
Volume : 0.5 ml/vial
Storage : Refrigerate at 2-8°C
Stability : 12 months in unopened vial or as indicated on label. Once opened, the control should be used within 14 days or aliquoted and stored frozen. Avoid multiple freezing and thawing cycles.

7. Wash Buffer Concentrate - Requires Preparation.
Contents : One bottle containing buffer with a non-ionic detergent and a non-mercury preservative.
Volume : 50 ml/bottle
Storage : Refrigerate at 2-8°C
Stability : 12 months or as indicated on label.
Preparation : Diluted 1:10 in distilled or deionized water before use. If the whole plate is to be used dilute 50 ml of the wash buffer concentrate in 450 ml of water.

8. Assay Buffer - Ready to use.
Contents : One vial containing a protein-based buffer with a non-mercury preservative.
Volume : 15 ml/vial
Storage : Refrigerate at 2-8°C
Stability : 12 months or as indicated on label.
9. **TMB Substrate** - Ready to use.
Contents : One bottle containing tetramethylbenzidine and hydrogen peroxide in a non-DMF or DMSO containing buffer.
Volume : 16 ml/bottle
Storage : Refrigerate at 2-8°C
Stability : 12 months or as indicated on label.

10. **Stopping Solution** - Ready to use.
Contents : One vial containing 1M sulfuric acid.
Volume : 6 ml/vial
Storage : Refrigerate at 2-8°C
Stability : 12 months or as indicated on label.

**Procedure**

All reagents were kept room temperature before use. Calibrators, controls and specimen samples should be assayed in duplicate. Once the procedure started, all steps should be completed without interruption.

Working solution of the estrone conjugate and wash buffer were prepared and removed the required number of microwell strips. The bag was resealed and unused strips were returned to the refrigerator. Pipetted 50 μl of each calibrator, control and specimen sample into correspondingly labelled wells in duplicate. Pipetted 100 μl of the conjugate working solution into each well (We recommend using a multichannel pipette). Incubated on a plate shaker (approximately 200 rpm) for 1 hour at room temperature. Washed the wells 3 times with 300 μl of diluted wash buffer per well and tapped the plate firmly against absorbent paper to ensure that it is dry (The use of a washer is recommended). Pipetted 150 μl of TMB substrate into each well at timed intervals. Incubated on a plate shaker for 10-15 min at room temperature (or
until calibrator A attains dark blue color for desired OD). Pipetted 50 μl of stopping solution into each well at the same time intervals as in step 7. Read the plate on a microwell plate reader at 450 nm within 20 min after addition of the stopping solution.

* If the OD exceeds the upper limit of detection or if a 450 nm filter is unavailable, a 405 or 415 nm filter may be substituted. The optical densities would be lower; however, this will not affect the results of patient/control samples.

**CALCULATIONS**

1. Calculate the mean optical density of each calibrator duplicate was calculated.
2. A calibrator curve on semi-log paper with the mean optical densities on the Y-axis was drawn and the calibrator concentrations on the X-axis. If immunoassay software is being used, a 4-parameter curve is recommended.
3. The mean optical density of each unknown duplicate was calculated;
4. Read the values of the unknowns directly off the calibrator curve.
5. If a sample read more than 2000 pg/ml then it was diluted it with calibrator A at a dilution of no more than 1:8. The result obtained should be multiplied by the dilution factor.

1. **SALIVARY PREGNANEDIOL-3-GLUCURONIDE**
   **(O'connor et al., 2003)**

   The procedure of the reagent preparation were the same as the steps indicated for the preparation of the reagents of salivary estrone-1-glucuronide [Folan et al, 1989].

Capture Ab-Quidel 330 Monoclonal
Signal-Horseradish Peroxidase-PDG (Used as standard)
Time needed to perform assay: 3 days
Preparing plates: Approximately 24 hrs minimum (coating overnight or several days at 4°C)
Remaining steps: 2 days

**Procedure**

Plates were coated with Quidel 330 Monoclonal antibody. After incubation (overnight or several days at 4°C), BSA blocking solution was added. Incubated plates 30 min, to 3 hrs at room temperature. After that standard, samples and controls were added. Preparation for this step required a minimum of 3 to 4 hrs, more if many plates are to be done at once. PDG-HRP was added immediately and then plates were incubated overnight at 4°C. After washing plates, substrate solution was added. After 15 to 20 min of incubation on a plate shaker, which continued to read the plates until the optical density of 2.0-2.9 ng/ml.

7). **QUANTITATIVE DETERMINATION OF THYROID HORMONE AND ITS METABOLITES USING RADIO IMMUNO ASSAY.**

1. **Salivary T3 (Larsen, 1972)**
   i) Goat Anti-Mouse IgG Coated micro titer wells, 96 wells
   ii) Enzyme Conjugate Concentrate (11x), 1.3 ml.
   iii) Enzyme Conjugate Diluent, 13 ml.
   iv) T3 Reference Standards, 0, 0.75, 1.5, 3.0, 6.0 and 10.0 ng/ml, 1 set, 1.0 ml each, ready to use.
   v) Antibody Reagent, 7 ml.
   vi) TMB Reagent (One-Step), 11 ml.
   vii) Stop Solution (1N HCl), 11 ml

2. **Salivary T4 (Skelley, 1973)**
   i) Sheep anti-T4 coated micro titer wells, 96 wells.
   ii) T4 Reference Standards: 0, 2, 5, 10, 15, and 25 ug/dl, 1 set, 1.0 ml, ready to use.
iii) Enzyme Conjugate Concentrate (11×), 1.3 ml.
iv) Enzyme Conjugate Diluent, 13 ml.
v) TMB Reagent (One-Step), 11 ml.
vi) Stop Solution (1N HCl), 11 ml.

3. Salivary TSH (Berger and Quinn, 1976)
i) Murine Monoclonal Anti-TSH-coated micro titer wells.
ii) Set of Reference Standards: 0, 0.5, 2, 5, 10 and 25 µIU/ml, lyophilized.
iii) Enzyme Conjugate Reagent, 13 ml.
iv) TMB Reagent (One-Step), 11 ml.
v) Stop Solution (1N HCl), 11 ml.

REAGENT PREPARATION
1. All reagents were allowed to reach room temperature (18-25°C) before use.
2. To prepare working T3-HRPO Conjugate Reagent, 0.1 ml of T3-HRPO conjugated concentrate (11×) was added to 1.0 ml of T3. The conjugated diluent (1:10 dilution) was mixed well.

Note: Prepared only the amount of Conjugate that was required each time. Working Conjugate Reagent was used within 24 hr. The excess after use was discarded.

ASSAY PROCEDURE
1. The desired number of coated well in the holder was secured and data sheet with sample identification was prepared.
2. Pipetted 50 µl of standard, samples, and controls into appropriate wells.
3. Dispense 50 µl of the Antibody Reagent into each well. Mix thoroughly for 30 sec.
4. Added 100 μl of Working Conjugate Reagent into each well. Mix thoroughly for 30 sec. It is important to have a complete mixing in this step.
5. Incubated at room temperature for 60 min.
6. Removed the incubation mixture by flicking plate contents into a waste container.
7. Rinsed and flick the micro titer wells 5 times with distilled or deionized water.
8. Shake the wells sharply onto absorbent paper to remove residual water droplets.
9. Dispensed 100 μl TMB Reagent into each well. Gently mix for 10 sec.
10. Incubated at room temperature in the dark for 20 min without shaking.
11. Stopped the reaction by adding 100 μl of Stop Solution to each well.
   • It is important to make sure that the blue color changes to yellow color completely.
13. Read the OD at 450 nm with a micro titer reader within 15 min.

**CALCULATION OF RESULTS**

1. The average absorbance values (A450) for each set of reference standards, control, and samples were calculated.
2. It was constructed a standard curve by plotting the mean absorbance obtained for each reference standard against its concentration in ng/ml on linear graph paper, with absorbance on the vertical (y) axis and concentration on the horizontal (x) axis.
3. Using the mean absorbance value for each sample, the corresponding concentration of T3 in ng/ml from the standard curve was determined.
8). Salivary Cortisol - Direct Salivary EIA (Brock et al., 1978)

REAGENTS PROVIDED

1. Rabbit Anti-Cortisol Antibody Coated Microwell Plate-Break Apart Wells -
   Ready to Use.
   Contents: One 96 well (12x8) polyclonal antibody-coated microwell plate in a resalable pouch with desiccant.
   Storage: Refrigerate at 2-8°C
   Stability: 12 months or as indicated on label.

2. Cortisol-Horseradish Peroxidase (HRP) Conjugate
   Concentrate Required for preparation
   Contents: Cortisol-HRP conjugates in a protein-based buffer with a non-mercury preservative.
   Volume: 300 µl/vial
   Storage: Refrigerate at 2-8°C
   Stability: 12 months or as indicated on label.
   Preparation: Dilute 1:50 in assay buffer before use (40 µl of HRP in 2 ml of assay buffer). If the whole plate that was diluted with 240 µl of HRP in 12 ml of assay buffer, and the left over was discarded.

3. Cortisol Saliva Calibrators - Ready To Use.
   Contents: Six vials containing cortisol in a protein-based buffer with a non-mercury preservative. Prepared by spiking buffer with a defined quantity of cortisol.
   *Listed below are approximate concentrations, please refer to vial labels for accurate concentrations. Storage: Refrigerate at 2-8°C
   Stability: 12 months in unopened vials or as indicated on label. Once opened, the standards were used within 14 days or aliquoted and stored frozen. Multiple freezing and thawing cycles were avoided.
4. Control - Ready To Use.

Contents : One vial containing cortisol in a protein-based buffer with a non-mercury preservative. Prepared by spiking buffer with a defined quantity of cortisol and referred to vial label for expected value and acceptable range.

Volume : 0.6 ml/vial

Storage : Refrigerate at 2-8°C

Stability : 12 months in unopened vial or as indicated on label. Once opened, the control was used within 14 days or aliquoted and stored frozen. Multiple freezing and thawing cycles were avoided.

5. Wash Buffer Concentrate - Required Preparation.

Contents : One bottle containing buffer with a non-ionic detergent and a non-mercury preservative.

Volume : 50 ml/bottle

Storage : Refrigerate at 2-8°C

Stability : 12 months or as indicated on label.

Preparation : Diluted 1:10 in distilled or deionized water before use. The whole plate to be used was diluted 50 ml of the wash buffer concentrate in 450 ml of water.

6. Assay Buffer - Ready to Use.

Contents : One vial containing a protein-based buffer with a non-mercury preservative.

Volume : 15 ml/vial

Storage : Refrigerate at 2-8°C

Stability : 12 months or as indicated on label.

7. TMB Substrate - Ready to Use.
Contents: One bottle containing tetramethylbenzidine and hydrogen peroxide in a non-DMF or DMSO containing buffer.

Volume: 16 ml/bottle

Storage: Refrigerate at 2-8°C

Stability: 12 months or as indicated on label.

8. Stopping Solution - Ready to Use.

Contents: One vial containing 1M sulfuric acid.

Volume: 6 ml/vial

Storage: Refrigerate at 2-8°C

Stability: 12 months or as indicated on label.

ASSAY PROCEDURE

1. Working solutions of the cortisol-HRP conjugate and wash buffer were prepared.

2. The required number of microwell strips were removed and resealed the bag unused strips was placed in the refrigerator.

3. Pipetted 50 µl of each calibrator, control and specimen sample into correspondingly labelled wells in duplicate.

4. Pipetted 100 µl of the conjugate working solution into each well (We recommend using a multichannel pipette).

5. Incubated on a plate shaker (approximately 200 rpm) for 45 min at room temperature.

6. Washed the wells 3 times with 300 µl of diluted wash buffer per well and tap the plate firmly against absorbent paper to ensure that it is dry (The use of a washer is recommended).

7. Pipetted 150 µl of TMB substrate into each well at time intervals.

8. Incubated on a plate shaker for 15-20 min at room temperature (or until calibrator A attains dark blue color for desired OD).
9. Pipetted 50 µl of stopping solution into each well at the same time intervals as in step 7.

10. Read the plate on a microwell plate reader at 450 nm within 20 min after addition of the stopping solution.

* If the OD exceeded the upper limit of detection or if a 450 nm filter was unavailable, a 405 or 415 nm filter was substituted. The optical density was lower; however, this did not affect the results of patient/control samples.

**CALCULATIONS**

1. Calculated the mean optical density of each calibrator duplicate.

2. Drawn a calibrator curve on semi-log paper with the mean optical densities on the Y-axis and the calibrator concentrations on the X-axis. If immunoassay software is being used, a 4-parameter curve is recommended.

3. Calculated the mean optical density of each unknown duplicate.

4. Read the values of the unknowns directly off the calibrator curve.

5. If a sample reads more than 100 ng/ml then dilute it with calibrator A at a dilution of no more than 1:8. The result obtained should be multiplied by the dilution factor.
3.2.4 SALIVARY ELECTROLYTES

1) Determination of salivary sodium (Trinder, 1951)

Reagents

1. Magnesium uranyl acetate reagent:
   8 g of uranyl acetate, 30 g of magnesium acetate and 30 ml of glacial acetic acid were mixed with 150 ml of water and heat to dissolve, then boiled for 2 min, cooled and diluted to 200 ml with water and made up to 1 litre with absolute ethanol. Placing it in a brown bottle 1 ml of 1 percent sodium chloride was added. The mixture stand for several days until the precipitated triple acetate settled out. The supernatant solution was used

2. Potassium ferrocyanide 10% solution. Keep in brown bottle

3. Acetic acid 1% (v/v)

4. Standard sodium solution (200 mEq. per litre):
   Dissolved 1.170 g of dry sodium chloride in water and made up to 100 ml.

Procedure:

5 ml of the magnesium uranyl acetate reagent was pipetted in each of two-glass stoppered centrifuged tubes. To one was added 0.5 ml of saliva from a pipette calibrated to contain and agitated the solution vigorously by blowing the pipette. To other the blanked one was used adding 0.5 ml of water, stopping for 5 min to allow formation of the precipitate of triple acetate is necessary. The tubes were shaken vigorously for 30 sec and centrifuged. 2 ml of the supernatant fluids (saliva) was measured into 100 ml. 80 ml of 1 % acetic acid along with 4 ml of 10% potassium ferrocyanide was added into volumetric flasks and made up to the mark with 1% acetic acid. After allowing 10 min for absorption the reading was noted at 480 nm using atomic absorption spectrophotometer.
2) Determination of Salivary Potassium (Jacobs and Hofmann, 1931)

Reagents

1. Sodium cobaltinitrate reagent (Kramer and Tisdall, 1921).
   Two solutions were prepared A and B.

   **Solution A:** 25 gms of cobaltous nitrate was dissolved in 50 ml of water, and added to the solution 12.5 ml of glacial acetic acid.

   **Solution B:** 120 g of sodium nitrite (this should be potassium free) was dissolved in 180 ml of water. About 220 ml of solution was obtained.

   210 ml of solution B was added to all solution A in a fume cupboard. Evolution of nitric oxide occurred immediately and drew air through the reagent until all the gas had been drawn off. A few hours later the solution was kept in a refrigerator and filtered before using.

2. Ethanol, 70% (v/v)
3. Choline hydrochloride (1% solution)
4. Sodium ferrocyanide (2% solution)
5. Standard cobalt solution:

   Various cobalt salts had been used. 0.351 gm of cobaltous sulphate was dissolved in water and made up to a litre. The latter salt recommended by King *et al* (1942) was the most convenient owing to the hygroscopic nature of others. In any case the cobalt solution should be standardized against the standard potassium solution (5.0 mEq. per litre) put through as described for the test. 1ml of the above cobalt solution is equivalent to 0.0025 mEq. potassium. The cobalt solutions was kept well.
6. Stock Potassium standard (250m.Eq per litre):

2.177 gm of pure dry potassium sulphate was dissolved in water and made up to 100 ml.

7. Standard Potassium solution for use (5 mEq. per litre):

2 ml of stock standard to 100 ml was diluted with water.

Procedure:

1 ml of hydrolysate saliva was pipetted into a tapered centrifuged tube at 6 ml. 2 ml for sodium cobaltnitrite reagent was added slowly, shaking continuously. After waiting for 45 min, 2 ml of water was added and mixed the contents well and centrifuged for 15 min for 6000 rpm. The supernatant fluid was removed by inverting the tube and allowing to drain on a filter paper for a short time. 1 ml of water was rundown the sides of the tube, centrifuged for 5 min and again upturn and drained. Then it was run in 2 ml of 70% ethanol and mix with the precipitate by means of a stirring rod centrifuge for 5 min invert and drain. The washing with ethanol was repeated.

After the final draining 2 ml of water was added and placed in a boiling water bath for 10 mins, shaking frequently to dissolve the precipitate. A small amount of insoluble material, probably protein may be left, but this did not interfere. It was allowed to cool, and first 1ml of 1% choline hydrochloride was added and then 1 ml of 2% sodium ferrocyanide, making up to the 6 ml mark. At the same time 2 ml of the cobalt standard was treated in the same way, again making up to 6 ml. reading was taken at 620 nm.
3) **Total phosphate in human saliva** (Gomorri, 1942)

Reagents

1. Trichloroacetic acid 10%, w/v solution in water.

2. Ammonium molydbdate solution:
   
   7.5 g of ammonium molydbdate was dissolved in about 200 ml of water added 100 ml of 10 N sulphuric acid and made up to 400 ml with water.

3. Metol (p-methyl aminophenol sulphate):
   
   1 g in 100 ml of 3% solution of sodium bisulphate.

4. Standard phosphate solution:
   
   0.2197g of potassium dihydrogen phosphate was dissolved in water, made up to a litre and added a few drops of chloroform.

**Procedure:**

0.8 ml of hydrolysate human saliva of 10 % trichloracetic acid was mixed well and filtered or centrifuged. Set up three tubes containing respectively 5 ml of the filtrate were set up to the saliva; 0.5 ml of standard plus 4.5 ml of 10% trichloroacetic acid was added with 5 ml of the trichloroacetic acid (blank). To each was added 1 ml of ammonium molydbdate solution and 1 ml of metol solution. This was allowed to stand for 30 mins and reading was taken at 680 nm using a red filter against the blank.

4) **Magnesium Determination in human saliva** (Neill and Neely, 1956)

Reagents

1. Sodium tungstate, 10% solution

2. Sulphuric acid, 2/3 N
3. Gum ghatti: 0.1 %

0.1 gm of powdered gum ghatti in a muslin bag was suspended in 100 ml of distilled water for twenty four hours. The solution was kept well at room temperature.

4. Titan yellow: 0.5 % Solution

0.1 gm of the powdered dye was dissolved in 200 ml of distilled water.

5. Sodium Hydroxide: 4N

6. Stock standard solution, containing 1mg/ml.

8.458 gm of MgCl₂.6H₂O was dissolved in distilled water and made up to 1 litre; or 10.094 gm of Mg.NH₄PO₄.6H₂O in 0.1N hydrochloric acid and made up to a litre, or dissolved 1.658 gm of reagent grade Magnesium oxide heated to constant weight at red heat, in 10 to 20 ml. of concentrated hydrochloric acid and make up to a litre with water.

7. Standard solution for use

1 ml of stock solution was diluted to 200 ml with water. This contains 5μg magnesium per ml.

8. Calcium Chloride solution: 0.05 mg calcium per ml.

13.88 mg of calcium chloride, CaCl₂ was dissolved in water and made up to 1 litre.

Procedure:

1 ml of hydrolysate saliva and precipitate proteins were diluted by adding with 2 ml of 10% sodium tungstate and 2ml of 2/3N sulphuric acid. Centrifugation to 0.5 ml of the supernatant fluid was added in turn with 1 ml distilled water, 1ml of the gum ghatti, 1ml of 0.05 percent, titan yellow and 2 ml of 4N sodium hydroxide. At the same time put up 1 ml of calcium chloride and 5
ml of water, and 1 ml of calcium chloride and 2.5 ml of standard for use plus 2.5 ml of water as blank and standard respectively, completing these in the same way as the test. The standard and unknown against the blank was read, using green filter or with the instrument set at 520 nm. Specially cleaned glassware was used.

5) Determination of calcium (Baron and Bell Method, 1959)

Reagents
   Mixed 20 ml of 0.1 M glycine (1.5 gm per 200 ml) and 80 ml of 0.1 M sodium hydroxide.

2. Ethylenediamine tetracetate (EDTA) disodium salt solution:
   0.93 gm of the disodium salt of EDTA was dissolved in water making the volume up to 1L.

3. Stock Standard solution (100 mg %):
   Weighed 2.5 gm of analar calcium carbonate, previously dried at 105°C for 24 hours, transferred it to a liter flask, added 200 ml water and 50 ml of normal hydrochloric acid. Allowed it to stand over night to dissolve and made up to the mark of 1L.

4. Working standard (10 mg %):
   10 ml of the standard solution was diluted to 100 ml with distilled water.

5. Calcein-thymolphthalein indicator:
   0.2 gm of calcein, 0.12 g of thymolphthalein and 20 gm of potassium chloride were grinded together in a mortar to get fine powder.
Procedure

1 ml of saliva was pipetted into 5 ml of buffer solution in a small china dish and added a small amount of the indicator (about 1 mg). Titrated with the EDTA solution using a micro-burette graduated to 0.01 ml. the end point was reached in aqueous solution when the color changed from yellow-green to mauve; and with saliva, from orange-green to pink. The color was stable for a few seconds, so titration was carried out fairly quickly. Titrate 1 ml standard and 1 ml of water (as blank) in the same way. The unit was expressed in mg/ml.

6) Determination of urea (Friedman, 1953)

Reagents
1. Sodium tungstate, 10%
2. Diacetyl monoxime reagent 2% solution in 2% acetic acid:
   
   2 gm of diacetyl monoxime was added to 60 ml of water followed by the addition of 2 ml of glacial acetic acid. It was shaken to dissolve with slight warming if necessary, and then made up to 100 ml.
3. Sulphuric acid-Phosphoric acid reagent:
   
   150 ml of 85% phosphoric acid was added to 140 ml water, mixed well and added 50 ml of conc. sulphuric acid slowly while mixing.
4. Sulphuric acid, 2/3 N
5. Stock standard Urea Solution:
   
   250 mg of urea was dissolved in 100 ml water. Working standard: diluted 1ml of stock to 200 ml with water (0.0125 mg/ml).

Procedure

For deproteinisation, 0.5 ml saliva and 3.3 ml of distilled water were taken and mixed well. 0.3 ml of sodium tungstate was added followed by 0.3 ml of 2/3 N sulphuric acid and mixed
thoroughly and kept aside for 10 min. The mixture was centrifuged to get a clear protein-free supernatant. Mixed well three tubes and placed in boiling water bath for 15 min cool and the readings was taken at 480 nm. The values were expressed in mg/ml.

3.2.5 SALIVARY SIALIC ACIDS AND GLYCOSAMINOGlyCANS

i) Thiobarbituric Acid Assay of Sialic acids (Warren, 1959)

Reagents

1. Sodium periodate (Meta) 0.2 M in 9 M phosphoric acid
2. Sodium arsenite, 10 per cent, in a solution of 0.5 M sodium sulfate-0.1 N H₂SO₄
3. Thiobarbituric acid, 0.6 per cent, in 0.5 M sodium sulfate.

All of these aqueous solutions were prepared with warming. Solutions were stored at room temperature and were stable for more than a month.

Procedure

To a saliva sample containing up to 0.05 pmole of N-acetylneuraminic acid in a volume of 0.2 ml was added 0.1 ml of the periodate solution. The tubes were shaken and allowed to stand at room temperature for 20 minutes. Arsenite solution, 1 ml, was added and the tubes were shaken until a yellow-brown color disappears. Thiobarbituric acid solution, 3 ml was added; the tubes were shaken, capped with a glass bead, and then heated in a vigorously boiling water bath for 15 minutes. The tubes were then removed and placed in cold water for 5 min. While cooling the red color faded and the solution often became cloudy. This did not affect the final reading. Of this solution, 1 ml was transferred to another tube which contained 1 ml of cyclohexanone. If desired, the entire 4.3 ml of aqueous solution could be extracted with 4.3 ml of cyclohexanone. The tube was shaken twice and then centrifuged for 3 minutes in a clinical centrifuge. The clear upper
cyclohexanone phase was red and the color was more intense than it was when in water. Optical densities of the organic phase were determined at 549 nm. The units were measured in mg/ml.

**ii) Determination of Salivary Glycosaminoglycans (GAGs) by using Alcian Blue 8GX** (Whiteman, 1973).

**Reagents**

1. Alcian Blue 8-GX (0.05% w/v)
2. Sodium Acetate (50mM, pH 5.8)
3. Acetic acid
4. Absolute Ethanol
5. 7.5% SDS (w/v)

**Procedure**

GAGs concentrations were determined from saliva according to the method of Whiteman (1973) which involves complex formation was mixed with 1 ml of a reagent containing 0.05% (w/v) Alcian Blue 8GX and 50mM-MgCl$_2$ in 50mM-sodium acetate adjusted to pH 5.8 with acetic acid. After equilibration for 2 hr at room temperature, the glycosaminoglycan-Alcian Blue complex was separated by centrifugation at 2000 g for 15 min. After the precipitate had been washed with ethanol (2 ml) it was dissociated with 1 ml of 40% (w/v) Manoxol IB solution. The resulting clear blue solution was measured in 1 cm microcuvettes with UV-spectrophotometer at 620 nm. The glycosaminoglycan contents of saliva samples were determined by reference to a calibration curve constructed by using chondroitin-4-sulphate as standard. The results were conveniently expressed as mg/ml of saliva.
3.2.6 LIPIDS AND ITS CONSTITUENTS

i) **LIPID SEPARATION**

Procedure

Total lipid was estimated using Folch *et al.*, (1957) methodology. 1 ml of saliva was taken and mixed thoroughly in 2 ml of chloroform and methanol mixture (2:1 v/v) in which 1 ml of 0.9% sodium chloride was added. This was allowed to stand for few hours. The lower phase was separated and chloroform was added to make up the original volume. This solution was dried under vacuum desiccators over silica gel and the residue was placed in boiling water bath for 10 minutes. After boiling, the mixture was cooled at room temperature from that 0.5 ml of acid digest was taken in a clean test tube and 5 ml of vanillin reagent was added, then it was mixed well and incubated for 30 min. When the pink color developed it was read at 530 nm. Blank was prepared by adding 5 ml of vanillin reagent to 1 ml of distilled water. The amount of lipid was quantified out using cholesterol standard graph. Total lipid content was expressed as mg/ml of saliva.

ii) **FATTY ACID PROFILE**

5 ml of saliva was taken and 1ml of reagent A was added in a teflon lined screw cap tubes. The tubes were tightly closed and kept for 30 minutes at 100°C in a water bath. 2 ml of reagent-B was added to each tube and kept again in a water bath at 80°C for 20 minutes. The tubes were closed down to the ambient temperature. Reagent -C (1.25 ml) was added to each tubes, and then closed tightly, shaken thoroughly for 10 minutes. About 2/3 of the organic phase (upper layer) containing the fatty acid methyl esters were transferred into screw cap glass vials. From each vial 1 μl of the fatty acid methyl ester (FAME) was injected into the gas chromatography (GC) column (Miller and Berger, 1985).
**GC conditions**

<table>
<thead>
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<th>Parameter</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>DEGS (Diethylene Glycol Succinate)</td>
</tr>
<tr>
<td>Oven temperature</td>
<td>180 °C (Isothermal)</td>
</tr>
<tr>
<td>Injection temperature</td>
<td>200 °C</td>
</tr>
<tr>
<td>Detector</td>
<td>FID (Flame ionization detector)</td>
</tr>
<tr>
<td>Detector temperature</td>
<td>210°C</td>
</tr>
<tr>
<td>Carrier gas</td>
<td>Nitrogen 30 ml/min.</td>
</tr>
</tbody>
</table>

The individual fatty acid was identified and quantified by using the FAME standard under similar conditions. The results were in mg/ml of lipid sample.

**Reagents:**

1. Saponification reagent
   
   45 g of NaOH in 300 ml of methanol was dissolved in the water mixture (1:1 v/v).

2. Methylation reagent
   
   Methylation reagent was prepared by mixing 325 ml of 6N HCl with 275 ml of methanol.

3. Extraction solvent
   
   200 ml of hexane was mixed with 200 ml of anhydrous diethyl ether.

4. Base wash
   
   10.8 g of NaOH was dissolved in 900 ml of distilled water.

iii) **Free fatty acids was estimated by the method of** Horn and Menahan (1981)

Reagents:

2. Copper nitrate-triethanolamine solution (Cu-TEA) reagent:
   50 ml of 0.1 M copper nitrate and 50 ml of 0.2 M triethanolamine were mixed with 33 g of sodium chloride
3. Diethyl dithiocarbamate solution: 0.1% in n-butanol
4. Activated Silicic acid: Silicic acid was washed with 4N HCl and then with distilled water until the pH was neutral. After drying, both were added in sufficient quantity, stirred well and the supernatant was decanted. This was then dried at 60°C and activated at 100°C overnight prior to use.
5. Standard Palmitic acid: 20 mg of palmitic acid in 100 ml of chloroform.

Procedure:
To 0.2 ml of Folch-wash aliquot/saliva, 6.0 ml of chloroform-heptane-methanol mixture was added and shaken vigorously. 200 mg of activated silicic acid was added shaken and left aside for 30 mins. The solution was then centrifuged and the supernatant was transferred to a tube containing 2.0 ml of Cu-TEA reagent. Blank contained only the solvent while the standard had different concentration of palmitic acid made up to known volume with the solvent and then 2.0 ml of Cu-TEA solution was added. The contents were agitated using a mechanical shaker for 20 min. The mixture was separated into two phases by centrifugation. 2.0 ml of the upper phase was transferred into another tube and mixed with 1.0 ml of diethyl dithiocarbamate solution. The yellow color developed was read at 430 nm. Free fatty acid was expressed as mmol/L.

iv) Total cholesterol was estimated by the method of Zlatkis (1935)
Reagents:
   1. Ferric chloride-acetic acid reagent- 0.05%
   2. Conc Sulphuric acid
3. Cholesterol working standard: 40 \( \mu g/ml \) in ferric chloride acetic acid reagent.

Procedure:

To 0.1 ml of the sample lipid extract, 9.9 ml of ferric chloride-acetic acid reagent was added and allowed to stand for 15 minutes and then centrifuged. To 5 ml of the supernatant, 3.0 ml of concentrated sulphuric acid was added. The color developed was noted after 20 min at 560 nm against a reagent blank. Values were expressed as mmol/L.

v) Phospholipids were estimated by the method of Rouser et al., (1970)

Reagents

1. TCA 10% was mixed in distilled water
2. Perchloric Acid 70%
3. Ammonium molybdate 3%: Three grams of ammonium molybdate was dissolved in 100 ml of distilled water.
4. Ascorbic acid 3%: Three grams of ascorbic acid was dissolved in 100 ml of distilled water.
5. Standard Phosphorus: 35.1mg of potassium dihydrogen phosphate was dissolved in 100 ml of double distilled water to give a concentration of 80\( \mu \)gm of phosphorus/ml. A concentration of 8 \( \mu \)g/ml was prepared by diluting the stock solution from 1 to 10 ml with deionised water.

Procedure:

1.0 ml of saliva was made up to 2.0 ml with water. To this, 1.5 ml of TCA was added and centrifuged. The precipitate was dissolved in 1.0 ml of perchloric acid, digested on a sand bath till the solution becomes colorless. After cooling, the solution was made up to 5.0 ml with double distilled water. To all the tubes, 0.5 ml each of ammonium molybdate and ascorbic acid were added
and the mixture was kept in a boiling water bath for 6 min. The blue color developed was read at 620 nm using a UV spectrophotometer. Phospholipids content was expressed as mmol/L.

vi) **Triglycerides were estimated by the method of** Foster and Dunn (1973).

**Reagent:**

1. Isopropanol
2. Activated aluminium oxide (Neutral)
3. Saponification reagent: 5.0 g of potassium hydroxide was dissolved in 60 ml of distilled water and added 40 ml of isopropanol.
4. Sodium Meta periodate reagent: 77 g of anhydrous ammonium acetate was dissolved in about 700 ml of distilled water. To this 60 ml glacial acetic acid was added followed by 650 mg of sodium metaperiodate. The mixture was diluted to 1 liter with distilled water.
5. Acetyl acetone reagent: 0.75 ml of acetyl acetone was dissolved in 60 ml of distilled water and added 40 ml of isopropanol.

**Procedure:**

An aliquots of lipid extract was evaporated to dryness in which 0.1 ml of methanol was added followed by 4.0 ml of isopropanol. To this 0.4 g of alumina was added to all the tubes and shaken well for 15 min. After centrifugation 2.0 ml of the supernatant fluid was transferred to labelled tubes. For saponification, all the tubes were kept in a water bath at 65°C for 15 min after adding 0.6 ml of the saponification reagent and 0.5 ml of acetyl acetone reagent. After mixing, the tubes were placed in a water bath at 65°C for 1 hr. Then the contents were cooled and absorbance was read at 420 nm. A series of standards of
concentrations 8-40 µg triolein were treated similarly along with a blank containing only the reagents. All the tubes were cooled and read at 450 nm. The triglycerides content was expressed as mmol/L.

**vii) HDL-C were estimated by the method of** Whitehead *et al.*, (1979)

**Reagents**

1. **Heparin solution:**
   - 1 vial of sodium heparin was diluted with sodium chloride solution, 9 gm/l to give a final concentration.

2. **Manganese chloride solution:** (2.02 mol/L)
   - 40.1 gm MnCl₂.4H₂O was dissolved in water and made up to 100 ml.

**Procedure**

To 1 ml of saliva in a centrifuge tube was added with 0.1 heparin solution and vortex was mixed for 10 sec. 50 µl MnCl₂ was added and remixed similarly. This was placed in an ice bath for 30 min and then centrifuged 1500 rpm at 4°C for 30 min. The clear supernatant was carefully removed. If there is turbid recentrifuge at high speed to remove the pellet for high density lipoprotein analysis. Then analyzed for an enzymatic method bearing in mind the dilution factor of 1.10. For LDL equal to triglycerides minus HDL+VLDL. For VLDL assay Triglycerides divided by 5. The units are measured in mmol/L.

### 3.2.7 Salivary Volatiles determination using GC-MS Analysis

**SOLVENT SELECTION**

The solvent, dichloromethane (DCM) extraction showed maximum response in obtaining the compounds (Preti and
Huggins, 1976); therefore, the DCM was used as a solvent in the present study.

**SAMPLE ANALYSIS**

Each sample was thawed at room temperature prior to analysis. Then 1 ml of samples in triplicate of the human saliva was taken and separately mixed with 1 ml dichloromethane. The supernatant was filtered through a silica gel column (60-120 meshes) and concentrated under vacuum (temp. <30°C) for fractionation and chemical identification by gas chromatography linked mass spectroscopy (GC-MS) analysis.

**FRACTIONATION**

Salivary samples were taken from normal menstrual cycle at different reproductive phases and were stored at -20°C until further use. The samples were fractionated using a vacuum technique (Preti and Huggins, 1976). The samples (20 ml) were placed in a distillation flask and the volatile substances were distilled for 2 h at room temperature under a vacuum of 0.2 Torr. Cooling with liquid nitrogen the sample was condensed the distillate. To restore the one fourth of the original volume (5 ml) distilled extra pure solvent (i.e. the solvent which was used for extraction) was added to the distillation residue. The volatiles from the distilled fraction were subjected to GC and GC-MS analyses (Pause et al., 1997).

**GAS CHROMATOGRAPHY AND MASS SPECTROMETRY**

The GC-MS analyses were made in QP-5000, (Thermo Finnagan, Japan). The 2 μl of extract was injected into the GC-MS on a 30 m glass capillary column with a film thickness of 0.25 μm (30 m X 0.2 mm i.d. coated with UCON HB 2000) using the following temperature programme, initial oven temperature of 40°C for 4 minutes increasing to 250°C at a rate of 15°C for 10 minutes.
The gas chromatography (Thermo Finnagan) was equipped with FID detector connected to an integrator. The area under each peak was used for quantitative calculations. The detection accuracy was about 1ng/peak. The relative amount of each component was reported as the percent of the ion current. The GC-MS was under the computer control at 70-ev. Using ammonia as reagent gas at 95-ev was performed chemical ionization. Identification of unknown compounds was made by probability based matching using the computer library built within the NICT 12 system. The GC-MS operating programme is given below:

**GC-MS programme**

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<th>Name of GC-MS</th>
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<td>(Thermo Finnagan, Japan)</td>
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<td>GC-parameters</td>
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<tr>
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</table>
3.2.7 SALIVARY ENZYMES AND ANTIOXIDANT PROFILES

i) **Estimation of Alkaline phosphatase in human saliva**

(King and Armstrong, 1934)

Phosphatases were enzymes, which catalyze the splitting up of phosphoric acid from monophosphoric esters. Alkaline phosphatase acted on sodium β-glycero phosphate at pH- 10 to liberate inorganic phosphorus. This phosphorus was allowed to react with molybdic acid to give phosphomolybdate, which in turn was reduced by 1-amino-napthol, 4-sulphonic acid (ANSA) to molybdenum blue.

Reagents:

1. Substrate: 0.1 ml sodium β-glycero phosphate was dissolved in 100 ml

2. Sodium carbonate (Bicarbonate buffer 0.1ml, pH- 10)

3. 10% TCA

4. Ammonium molybdate: 2.5% in 3N Sulphuric acid
5. ANSA: 500 mg of ANSA was dissolved in a mixture of 195 ml of 15% sodium bisulphate and 5 ml of 20% sodium sulphite solution.

6. Standard Phosphorus: 35.1 mg potassium dihydrogen phosphate was dissolved in 100 ml glass distilled water 10 ml of this solution was distilled to 100 ml to prepare a working standard containing 8 µg/ml phosphorus.

Procedure:
To make up a 1 ml of buffered substrate 0.2 ml of saliva was added and incubated at 37°C for 1 hr. The tubes were removed and 1 ml 10% TCA was added mixed and centrifuged for 10 min. To 1 ml supernatant, 1 ml of ammonium molybdate and 0.4 ml ANSA were added. The color developed was read in a UV spectrophotometer at 680 nm. A system devoid enzymes served as control. A series of standard in the concentration 0.156 to 0.781 µmoles were also processed similarly and the enzyme activity was expressed as IU/L.

ii) Estimation of Lactate dehydrogenase (King, 1965)

Reagents
1. Glycine buffer (0.1M)
   7.50 gm of glycine and 5.85 gm of NaCl were dissolved in distilled water and made up to 1 litre.

2. Buffered Substrate:
   125 ml of glycine buffer was added and 75 ml of 0.1N NaOH to 5 ml of sodium lactate solution (which is approximately 70%). Alternatively 4 gm of lithium lactate which is more reliable in composition than sodium salt can be used.
3. NAD solution:
   10 mg of NAD was dissolved in 2 ml of distilled water and kept at 0.4°C.

4. DNPH Reagent:
   200 mg of DNPH was dissolved in hot 1N HCl and made up to 1 litre with the same.

5. 0.4 N NaOH

6. Standard Sodium Pyruvate:
   11 mg of sodium pyruvate was dissolved in 100 ml of buffered substrate solution.

Procedure:

   1 ml of buffered substrate and 0.1 ml of saliva were placed (diluted 1:5 with distilled water) into two test tubes. 0.2 ml of distilled water was added to one tube (blank), placed both in a water bath at 37°C and allowed them to reach the bath temperature. To the other tubes 0.2 ml of NAD solution was added and mixed. After incubating for 15 min and then 1 ml of DNPH reagent was added to both tubes, mixed and kept in water bath for further 15 min. The tubes were removed and 10 ml of 0.4 N NaOH was added and read the OD at 440 nm within 1 to 5 min. The values were measured IU/L.

**iii) Estimation of Ascorbic acid** (Roe and Kuether, 1943; Roe, 1961)

Reagents

1. Trichloroacetic acid (6% TCA)

   About 6 gm of TCA was dissolved in 100 ml of 9 N Sulphuric acid.
2. 4% Thiourea Solution

44 gm of Thiourea was dissolved in 100 ml of distilled water.

3. 85% Sulphuric acid

About 85 ml of sulphuric acid was made up to 100 ml with distilled water.

4. Stock Standard Solution

100 mg of ascorbic acid in 100 ml of 4% oxalic acid (1mg/ml).

5. Working Standard Solution

10 ml of stock was made up to 100 ml with oxalic acid.

Procedure

To make up 6 ml of saliva, 6% TCA was added in a centrifuge tube and centrifuged 1200 rpm for 10 min. Then 1.0 ml supernatant was made up to 0.3 ml with distilled water. Then 1.0 ml of 2, 4 dinitro phenyl hydrazine reagents and 1.0 ml of thiourea solution were added. All tubes were heated in a water bath for about 10 min and cooled at room temperature. Simultaneously a blank containing 3.0 ml of distilled water, 1.0 ml of 2, 4 dinitro phenyl hydrazine and 1.0 ml of thiourea was heated along with standard. After cooling it 6.0 ml of 85% sulphuric acid was added. The color developed then was read at 540 nm. The values are measured in mg/ml.

iv) Determination of Uric acid (Caraway, 1955)

Reagents

1. 10% Sodium Tungstate
2. 2/3 N Sulphuric acid
3. 10% Sodium carbonate (W/V)
4. Phosphotungstic acid:
50 gm of sodium tungstate was dissolved in about 400 ml of water. Added 40 ml of 85% phosphoric acid and reflux was added for 2 hr. After cooling it was transferred to a 500 ml of flask and made to the mark with water and kept in brown bottle. There it was diluted 1 ml in 10 for use.

5. Tungstic acid:
50 ml 10% sodium tungstate, 50 ml 2/3 N sulphuric acid and a drop of phosphoric acid were added with 800 ml water. It is discarded when cloudy and kept in brown bottle for further use.

6. Stock Standard Uric acid: (100 mg/ml)
100 mg of uric acid and 60 mg of lithium carbonate were mixed and kept in a small beaker. Then it was dissolved in 15-20 ml of water in a test tube. Heated the solution to 60°C and poured on to the uric acid, and stirred until dissolved, heating further in warm water if necessary. When dissolved, transferred with washing to a 100 ml flask. Added 2 ml of 40% formalin and then slowly with shaking, added 1ml of 50% v/v acetic acid. Finally it was made to the mark with water, mixed and kept in well-stoppered bottle away from sunlight.

7. Working Standard:
For one point calibration, 1ml of stock to 200 ml was diluted with distilled water (1ml+0.005 mg). For standard curve, 1ml stock is diluted to 100 ml with distilled water (0.01 mg/ml).

Procedure

5.4 ml of dilute tungstic acid was added, shaken to 1ml of saliva and centrifuged into three test tubes, measured 3 ml of the
supernatant, 3 ml of the diluted standard and 3 ml of water as blank and mixed and placed in a water bath at 25°C for 30 min. The reading was taken in optical density at 650 nm (red filter) and the values were expressed in mg/ml.

v) Determination of Peroxidase activity in human saliva by (Putter, 1974)

Reagents

1. Phosphate buffer 0.1 M (pH-7.0)
2. Guaiacol Solution 20 mM:
   240 mg guaiacol was dissolved in water and made up to 100 ml. It was stored frozen for many months.
3. Hydrogen peroxide solution (0.042%= 12.3 mM).
   0.14 ml of 30% H₂O₂ was diluted to 100 ml with water. The dilution factor of this solution should be 0.485 at 240 nm, and this was prepared afresh every time for further use.
4. Enzyme Extraction:
   1 ml of saliva was added with 3 ml of 0.1 M phosphate buffer pH- 7.0. Then it was centrifuged at 18,000 g at 5°C for 15 min. The supernatant was used as enzyme source within 2-4 hr and stored on ice till the assay was carried out.

Procedure:

3 ml buffer solution, 0.05 ml guaiacol solution was pipetted, and 0.1 ml enzyme was extract and 0.03 ml hydrogen peroxide solution was mixed in cuvette bring the buffer solution to 25°C before assay. Mixing well the cuvette was placed in the spectrophotometer and waited until the absorbance has increased by 0.05. The time required was noted using stop-watch to increase
the absorbance by 0.1 was noted. The values are measured in mmol/L.

**vi) Estimation of Gluthathione peroxidase (EC 1.11.1.9)**

(Rotruck *et al.*, 1973)

**Reagents**

1. Tris-Hcl Buffer: 0.4M; pH 7.0
2. Sodium Azide solution: 10mM
3. 10% TCA
4. EDTA: 0.4mM
5. H₂O₂: 1.0mM
6. Glutatione Solution (GSH): 2mM

**Procedure**

To 0.2 ml of Tris buffer, 0.2 ml of EDTA, 0.1 ml of sodium azide and 0.2 ml of enzyme preparation (haemolysate) were added and mixed well. To this 0.2 ml of GSH followed by 0.1 ml of H₂O₂ were added to contents, mixed and incubated at 37°C for 10 mins along with control connecting all reagent except enzymes. After 10 minutes the reaction was arrested by the addition of 0.5 ml of 10% TCA. The tubes were centrifuged and the supernatants were assayed for GSH. The activities were expressed as µg of GSH consumed mmol/L in saliva.

**vi) Assay of Catalase (EC 1.11.1.6)** (Sinha, 1972)

**Reagents**

1. Phosphate buffer (0.01M, pH 7.0)
2. Hydrogen peroxide (0.2M)
3. Potassium dichromate (5%)
4. Potassium dichromate-Acetic acid (1:3 ratio):
The ratio of potassium dichromate-acetic acid was mixed. From this 1.0 ml was diluted again with 4.0 ml of acetic acid.

5. Standard $H_2O_2$:

$0.1 \text{ml of } 0.2M \, H_2O_2$ was diluted to 100 ml using distilled water.

Procedure

To 0.9 ml of phosphate buffer, 1 ml of saliva and 1 ml of hydrogen peroxide were added. After 60 sec 2.0 ml of potassium dichromate-acetic acid ratio was added. The tubes were kept in boiling water bath for 10 mins and the color developed was read at 620 nm. Standards (2-10 $\mu$mol) were taken and preceded as test with blank containing reagent alone. The activity was expressed as $\mu$M of $H_2O_2$ consumed mmol/L.

vii) **Determination of Superoxide dismutase (EC 1.15.1.1)**

(Marklund and Marklund, 1947)

Reagents

1. Tris-Hcl: 0.05 $P^h$ 7.4
2. Pyrogallol stock standard
   
   25.6 mg of pyrogallol was dissolved in 1.0 ml of 0.05M Tris buffer.
3. Pyrogallol Working Standard
   
   1.0 ml of stock was diluted to 100 ml using 0.05 M Tris buffer.
4. Absolute Ethanol
5. Tris-Hydrochloric acid buffer: 0.1M $P^h$-8.4
Procedure

To 0.5 ml of clear homogenate, 0.25 ml of ice-cold chloroform, 0.15 ml of ice-cold ethanol and 1.0 ml of water were added, the solution was shaken thoroughly and centrifuged at 12,000 rpm for 15 min. The supernatant was used for enzyme assay. To this 0.5 ml of the supernatant, 2.0 ml of Tris buffer (0.1M) and 1.5 ml of water were added. The reaction was initiated by the addition of 0.5 ml of pyrogallol reagent and the change in optical density which was measured at 470 nm for 3 min. The enzyme activity was expressed as units mmol/L.

3.2.8 SALIVARY AMINO ACIDS

Total Amino acids (Acid hydrolysis)

1 ml aliquots of saliva were deproteinized with 50 mg sulfosalicyclic acid (Sigma st, Louis, USA) and centrifuged was done for 10 min at 4000 x g at 4° C. The pellet was washed with distilled water. A known weight of the pellet was transferred to a hydrolysis tube. 5 ml of 6 N HCl was added to the pellet and incubated the tube at 110°C for 18 hr. Transfer the contents to a china dish after hydrolysis. Acid vapors were completely removed by keeping the china dish over a boiling water bath by repeated evaporation using distilled water. The residue was made up to a known volume and kept in the refrigerator.

The sample was extracted with 80% ethanol (if necessary the mixture was heated at 70-80°C for 30 min in a water bath) and centrifuged at 10,000 x g for 10 min. The clear supernatant was concentrated and used for the assessment of amino acids. Standard amino acid was also run parallel with the unknown samples. From the standard profile, the amino acid concentration
of unknown sample was identified and quantified by reverse phase-HPLC (Van eijk et al., 1988).

3.2.9 SALIVARY PROTEIN ESTIMATION (Bradford, 1976)

Reagent Preparation

Bradford Reagent

Coomassie brilliant blue G250 - 100 mg
Ethanol 95% - 50 ml
Phosphoric acid 85% - 100 ml
Distilled water - 1000 ml

Procedure

Total protein content in the sample was estimated using Bradford method. The 20 μl of each sample was taken and mixed with 0.980 ml of distilled water and 2.5 ml of Bradford reagent. The test tubes were left for 20 min until the appearance of blue color, was measured at 595nm in UV visible Spectrophotometer (Ferkin elmer, Germany). A proper blank solution containing 1 ml of the distilled water and 2.5 ml of Bradford reagent were also prepared. The bovine serum albumin (BSA) was used as standard. The protein concentration was expressed in mg / ml of sample.

SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS [SDS-PAGE]

Principle

Electrophoresis is an analytical method used to separate, isolate and determine the molecular weight of the charged substance such as proteins and nucleic acids. Its is based on the fact that the electrical charge causing molecules migrate towards opposite electrode through the medium and became separated when it is subjected into electric field. The movement of particle in
electrophoresis is directly proportional to electric field strength and net charge of the molecule and inversely proportional to size of the molecular and frictional properly the medium is used.

Polyacrylamide gel was prepared by linear polymerizing agent acrylamide and a small quantity of cross linking reagent methylenebisacrylamide also made in the presence of initiator ammonium per sulphate and catalyst tetra methyl ethylene diamine [TEMED].

SDS-PAGE was carried out following the method of Laemmli (1970). The proteins denatured by SDS in the presence of reducing agent β mercaptoethanol and converted to a rod like lined polypeptide. These molecules when subjected to electrophoresis migrate according to their molecular size and weight.

Reagents required

<table>
<thead>
<tr>
<th>Monomer solution:</th>
<th>30% acrylamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>-14.6g</td>
</tr>
<tr>
<td>Bis. Acrylamide</td>
<td>-0.4g</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>made up to -50ml</td>
</tr>
<tr>
<td>Store at 4°C in the dark room.</td>
<td></td>
</tr>
</tbody>
</table>

Running gel buffer [1.5 m Tris-HCL pH 8.8]

| Tris                | -9.075g        |
| Adjust the pH 8.8 with HCL |
| Distilled Water     | made up to -50ml |

Stacking gel buffer [1m Tris –HCL pH 6.8]

| Tris                | -3.029g        |
| Adjusted the pH 6.8 with HCL |
| Distilled water     | made up to -50ml |

10% SDS
SDS r2.5g
Distilled water made up to -25ml

10% Ammonium per sulphate
Ammonium per sulphate - 0.5 g
Distilled water made up to -5 ml

Tank buffer [0.025 m tris, pH 8.3, 0.192m glycine, 0.1% SDS]
Tris -1.5g
Glycine -7.2g
SDS -0.5g
Distilled water made up to -500ml

2x sample buffer [0.250 m Tris-HCL pH6.8, 4%. SDS, 20% glycerol, 10% mercaptoethanol]
Tris gel buffer [pH 6.8] -1.25 ml solution of stacking
SDS -2.0 ml solution of 10% SDS
Glycerol -1 ml
2-mercaptoethanol - 0.5 ml
Distilled water made up to -5ml
A pinch of bromophenol blue was indicator dye

Stain [0.125% coomassie blue R250, 10% acetic acid, 50% methanol]
Coomassie blue R 250 -200 mg
Acetic acid -7 ml
Methanol -50 ml
Distilled Water made up to -100 ml

Destaining solution I [50% methanol, 10% acetic acid]
Methanol -50 ml
Acetic Acid -10 ml
Distilled water made up to -100 ml

Destaining solution II [7% acetic acid, 5% methanol]
Acetic acid -7 ml
Methanol -5 ml
Distilled Water made up to -100 ml

Agar 1%
Agar -1 g
Distilled Water made up to -100 ml

Catalyst
NNNN- Tetra methyl ethylene diamine [TEMED]

Separating gel overlying solution
N-isobutanol -50ml
Distilled water -50 ml

Separating gel (12%)
Distilled Water -3.3 ml
30% acrylamide -4.0 ml
Tris pH 8.8 -2.5 ml
10% SDS -0.1 ml
10% APS -0.1 ml
TEMED -0.004 ml

Stacking gel (5%)
Distilled Water -2.1 ml
30% acrylamide -0.5 ml
Tris pH 6.8 -0.38 ml
10% SDS -0.03 ml
10% APS -0.03 ml
TEMED -0.003 ml

Preparation of gel
Two glass plates were sandwiched using 1.5 mm Teflon spacer strip and the sides and bottom were sealed with 1% agar. The separating gel (12%) mixture was poured between the two glass
plates then overlay with methanol and allowed for polymerization. The solution was then discarded after polymerization of the gel. After polymerization the Teflon comb was removed and the wells were rinsed with distilled water. After removing the basal strips the glass plates were fixed to the gel chambers.

**Preparation of the sample**

The protein concentrations of the samples were determined using a standard graph prepared adopting Bradford method. Samples containing protein was mixed with equal volume of sample buffer treated at 100°C for 3 minutes. The samples were then loaded into the wells of stacking gel. The chambers filled with tank buffer and electrophoresis started running. Initially a current of 50v was applied till the dye entered the separating gel sub sequentially current was increased to 100v. Electrophoresis was continued till the marked reached 1 cm above the edge of the separating gel.

**Staining and destaining gel**

After electrophoresis, the gel was removed from the glass plates and the bands in the gels were stained using staining solution for about 6 hours. Then the gel was destained using destaining solution until the background was clear.

**Determination of molecular weight by Gel documentation**

The molecular weight of the unknown fractions in the samples was determined by composing the Rf value of the unknown fractions with a standard graph drawn with molecular weight and Rf value for standard molecular weight marker. The molecular weights of the protein fraction were identified using quantity one gel documentation scanner and analysis software program, quantity one (Bio Rad, CA, USA).
3.2.10 Matrix Assisted Laser Desorption Ionization Time-of-flight Mass Spectrometry (MALDI-TOF MS)

SDS-PAGE

The 12% Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed after modified Laemmli (1970). Electrophoresis was carried out for 4 hrs in a constant voltage (50-v) at room temperature. Once the gel was completed it was rinsed with distilled water for 2 min and stained with 0.5% CBB R-250.

Protein detection by Coomassie Brilliant Blue (CBB) stain

Once the gel was completed it was rinsed with distilled water for 2 min and stained with 0.5% CBB R-250 which contained 500 mg of coomassie R-250, 40% methanol, 10% acetic acid for 2 hrs at room temperature. The stained gel was destained in solution containing 40% methanol and 10% acetic acid until the appropriate background was obtained.

Trypsin digestion

The trypsin in-gel digestion of salivary polypeptides was processed according to Amado, et al (2005). For in-gel digestion, bands from 17, 42, 68 and 91 kDa proteins were taken from the SDS-PAGE using a thin glass pipette and placed into micro centrifuge tubes. Each gel plug was destained using 100 µl of 50 mM ammonium bicarbonate, 50% (v/v) acetonitrile (1:1) and incubated at 37°C for 30 min. This step was repeated until no stain was visible in the gel spot. Gel pieces were sliced into small cubes, and placed in 1.5 ml eppendorf tubes pre-washed with ethanol. A blank control was obtained using a piece of gel cut in a blank region of the gel and processed in parallel with the sample bands.

The plugs were then incubated at 37°C with 50 µl of 10 mM DTT. After 30 min at 37°C the DTT was discarded and 50 µl of 55
mM iodoacetamide was added to each tube and incubated for 1 hr at room temperature in the dark. The iodoacetamide solution was then discarded and the plugs were washed twice as above before being dehydrated in 100 % acetonitrile and then rehydrated in 9 μl of 50 mM ammonium bicarbonate. After 30 min incubation at room temperature in dark, the gel particles were washed with 50 % acetonitrile in 0.1 M NH₄HCO₃ and dried in a Speed Vacuum evaporator. The dried gel pieces were swollen in a minimum volume of a 10 μl digestion buffer that contained 50 mM NH₄HCO₃, 5 mM CaCl₂, and 10 μl of trypsin (Promega, sequencing grade) in order to keep the gel pieces wet during enzymatic cleavage at 37°C overnight.

**MALDI-TOF/Mass Spectrometry (MALDI-TOF/MS)**

The samples (Tryptic fragmented) were prepared by mixing equal amounts (2:2) of peptide mixture with the matrix solution (α-Cyano-4-hydroxycinnamic acid) saturated with 0.1% TFA and acetonitrile (1:1). Then, the samples were analyzed in reflectron mode with delay time of 90 ns and 25Kv accelerating voltage in the positive ion mode. To improve the signal to noise ratio summation of 300 laser shots were taken for each spectrum. External calibration was done using peptide I calibration standard with masses ranging from 1046-3147 Da. Mass spectra were acquired using ULTRAFLEX-TOF/TOF mass spectrometer (Bruker Daltonics, Germany), equipped with a 337 nm pulsed nitrogen laser. MS-MS spectra were acquired by selecting the precursor mass with 8 Da window.

Spectra were processed using FLEX analysis software. Monoisotopic peptide masses were assigned and used in the database search. The protein identification was accomplished utilizing the MASCOT data base search engine (Matrix Science, London, UK) (http://www.matrixscience.com, search engine).
Scores were considered to be higher than the significant (P<0.05) level in the mascot search. Hence, proteins identified with scores less than the significant level were reported as unidentified.