MATERIALS AND METHODS

3.1 Materials and Reagents

Standard BPA of >97% purity was purchased from HiMedia. The stock solution was prepared in methanol and used for further dilutions. The methanol of HPLC grade was also obtained from HiMedia. Solution of 500ppm was made as stock solution by adding BPA in methanol. Further external dilutions of concentrations 50, 100, 200 and 250 mg/L were made by adding BPA free-water treated through Milli-Q water system (Millipore Corporation, USA).

3.2 Determination of Migration of BPA in Polycarbonate containers and Cans

3.2.1 Sample Collection A total of 15 samples were taken for the study to check migration of BPA which include baby feeding bottles, drinking water bottles, microwave safe polycarbonate bowl and cans. These containers were taken as these are the most commonly available in the Indian markets and are used frequently on daily basis by the population. Eight polycarbonate feeding bottles were taken under five different brand names (Brand A-E). These brands had the origin of different countries and the bottles were also imported from different countries. The names of brands have been given Table 1. Of these eight polycarbonate feeding bottles, five bottles (FB 1-5) were newly purchased from the local market and the rest three used feeding bottles (FB 6-8) were donated by anonymous people. Used polycarbonate bottles were of same brand as the new bottles. All the used bottles showed visible wear and opaque discoloration and used approximately for a period between 3-12 months, although the bottles were described as to be used under normal conditions. The capacity of the feeding bottles ranged from 125-250 ml.

Four polycarbonate drinking water bottles were taken of a particular brand. Two of the drinking water bottles were newly purchased from the local market while two bottles were donated by anonymous people from the local area. The new polycarbonate bottles and the used bottles were of the same brand (Table 1). The used bottles showed some opaque discoloration due to regular use and were being used for more than six months. The roughness on the polycarbonate material
was due to the repeated washing of the bottles with brush and detergent. The capacity of the drinking water bottles ranged from 750-1000 ml.

A used microwave safe polycarbonate bowl of a particular brand (Brand F) was donated by an anonymous university student. The bowl was described to be used for the purpose of microwaving food and boiling water for approximately six months. The capacity of the microwave safe plastic bowl was approximately 350 ml.

Two samples of canned products were collected from a local supermarket. The two canned products were of different brands comprising different kind of food product. One can comprised of canned fruit (pineapple) (CN 1) and the other can comprised of canned green peas (CN 2). Both the canned products were made by different companies (Brand G and H) (Table 1). These canned products were taken as these are very commonly used by the local population and moreover, the people are progressively becoming more dependent on packed and canned products. Furthermore, the owners of local supermarkets were surveyed verbally about the most frequent sale of canned products, so these two types of canned food products were chosen for the study. These cans were stored in the supermarket under normal temperature conditions.

3.2.2 Sample Preparation

3.2.2.1 Polycarbonate feeding bottles

A standard washing procedure was followed for each sample of polycarbonate feeding bottles. The same washing procedure was followed as used in the households to imitate the steps followed by the users, so as to approximately determine the amount of BPA leach out in those samples. Each of the eight feeding bottles was filled with distilled water treated through Milli-Q water system and rinsed well. Detergent was then added into the bottles and they were shaken well for few times. Afterwards, these feeding bottles were washed by using a gentle brush. Again the bottles were rinsed with distilled water and then washed with Isopropyl alcohol to remove any residue. A final wash of distilled water was given five times to remove any residue of alcohol. The bottles were then air dried until completely. They were then kept in boiling water in a beaker for about 5-10 minutes. This step was followed to imitate the procedure of sterilizing
the feeding bottles as practiced among the local people at homes. The beakers used for boiling purpose were first rinsed with distilled water and kept in hot air oven at 200ºC to remove any water adsorbed on the surface of glass beaker. The bottles were then taken out of the beaker and filled with HPLC-grade water at 70ºC up to a certain level and kept for 1 hr with gentle shaking in between. After 1 hr, the water was poured into an autoclaved beaker and kept for boiling to evaporate the water. Approximately 5ml of water was reserved for further use and transferred into amber colored 5 ml vials and kept in refrigerator until used for HPLC analysis.

3.2.2.2 Drinking water bottles

For sample preparation of drinking water bottles, the bottles were washed following same procedure as described above except for the boiling step. After rinsing well the bottles with distilled water, these were air dried completely. The air dried bottles were then filled with HPLC-grade water at room temperature and kept for 24 hrs. After that, the water was transferred to a beaker and boiled for evaporation. 5ml water was reserved and stored in amber coloured glass vials in refrigerator until further use for HPLC-analysis.

3.2.2.3 Microwave bowl

For sample preparation of microwave bowl, the washing procedure was same as followed for feeding bottles. After air drying the bowl in a laminar air flow hood, HPLC-grade water was poured in the bowl and kept in the microwave for about 2-3 minutes at medium-high intensity for boiling. After that, the bowl was kept at room temperature for about half an hour. After that, the water was transferred into a beaker and boiled for evaporation. 5ml water was reserved and stored in amber coloured glass vials in refrigerator until further use for HPLC-analysis.

3.2.2.4 Canned products

For canned product samples, the products of the cans, both liquid and solid, were taken and put into the mixer for homogenization. 5ml of the semisolid product was taken out and poured into the polypropylene centrifuge tubes and 3 ml of dichloromethane was added and centrifuged at 3000 rpm for 10 minutes. The process was repeated three times. The removal of solvent was done by concentrating (1 ml) under reduced pressure at 60ºC. Then 0.5 ml of sulphuric acid was
added and the samples were again centrifuged at 3000 rpm for 10 min. To conclude, the organic phase was dried under nitrogen stream. The dried residues were suspended in 0.5 ml ethanol and stored in refrigerator until further use.

3.3 HPLC Analysis of BPA

3.3.1 Water samples

The water samples of feeding bottles, water bottle and microwave bowl were analyzed by HPLC-fluorescence. For compound identification of BPA, the standard solution and the external dilutions prepared were analyzed using Shimadzu HPLC System equipped with 2475 Pump, C$_{18}$ analytical column (Perkin Elmer, USA; Reverse Phase; 250 mm x 4.6 mm i.d.; 5 µm) and Fluorescence detector. Injection volume of 20µl was injected into the C$_{18}$ column. Isocratic elution was carried out with a mixture of methanol: water (v/v) in the ratio of 65:35 at a flow rate of 1ml/min with equilibration time of 10 min. The column temperature was set at 30ºC. The fluorescence detector was set at an excitation wavelength of 228nm and an emission wavelength of 313nm. The chromatograms were processed using Empower3 software.

3.3.2 Canned products

The canned product samples were also analyzed using the same HPLC-fluorescence system as described above for water samples analysis. However, the calibration was done at a flow rate of 1 ml/min with a 500 µl loop injector. Isocratic elution was done with n-hexane (phase A) for about 2 min, after a gradient was applied from 0 to 40% phase B (n-hexane: methanol: isopropanol at ratio of 40:45:15), 10 min to 100% phase B and 10 min to 100% phase A. The detector was set at a wavelength of 280 nm to monitor the elution profile. The chromatograms were processed using Empower3 software.
3.4 Survey and involvement of PCOS patients regarding their dependency on plastic containers and packed food and to study variation in their hormone levels

3.4.1 Survey of PCOS patients and healthy females regarding their dependency on plastic containers and cans

A survey was carried out involving both PCOS and healthy females to evaluate the dependency of both the groups on BPA containing polycarbonate products and packed food. A total of 150 females were being involved in the study aged between 13 to 45 years of reproductive age group. Of them, 100 PCOS females were surveyed from two different localities considering the factor of rural and urban population as it was assumed that the urban population is more dependent on packed foods and using polycarbonate containers for storage of food and drinks. The survey was carried out in University hospital by visiting the gynecology department. The rest of the patients were surveyed in the hospital of Amritsar (Punjab) where both rural and urban population visited the hospital. Similarly, for healthy females also, survey was done which included 50 females from both the regions also including university students.

The survey was carried out through a planned questionnaire of 34 questions which included various factors regarding their food habits and health. The study was approved by the Institutional Ethics committee of Maharishi Markandeshwar University. The purpose of the protocol was explicitted to all participants. A written informed consent was obtained from all the participants either from a legal guardian of each subject less than 18 years old or from those subjects 18 years old or older. Height and weight were measured in the hospital ward. Height was measured to the nearest inches by means of a fixed wall stadiometer. Body weight was determined in kilograms with a help of a calibrated balance beam scale.

Age, marital status, locality whether urban or rural, socioeconomic status, height, weight, body mass index (BMI), PCOS/healthy, hormone imbalance history and eating habits etc. along with the medical history were assessed through a self-made questionnaire. The BMI was calculated as weight (kg/height squared (m²)). The cut off values for the categorization of females under normal weight/over-weight/obese were as follows: 18.5-24.9 as normal weight, 25 to 29.9 as
over-weight and 30-34.9 as obese. Further information regarding the food habits and dependency of the participants on packed foods, canned food and usage of polycarbonate made plastic containers for food and water storage was given them which were filled in the questionnaire form by themselves or under their supervision. The information provided by them was kept completely confidential. The results obtained in the questionnaire were transferred into a tabular form where they were analyzed and compared with the results of healthy participants.

3.4.2 Involvement of PCOS patients and healthy female participants to study the variation in their hormone levels

Since BPA is a well-known endocrine disruptor, thought to be causing the hormone imbalance in the body, hence in this study, the hormone profiling of all the 100 PCOS patients was also done. The major hormones known to be involved in the PCOS are thyroid hormones- triidothyronine (T3), thyroxine (T4) and thyroid stimulating hormone (TSH), luteinizing hormone (LH), follicle stimulating hormone (FSH) and testosterone. These PCOS patients visiting the hospital for examination of the underlying disorder underwent these hormones profiling test. The hormone testing was done in the concerned hospital laboratory by the expert personnel by using ELISA for hormone analysis. The results for these tests were noted from test result reports. Simultaneously, the hormone profiling was also performed for the healthy females involved in the study. The results of the hormone test were noted down. The results of hormone profiling among both the groups were compared and further groups were divided accounting various factors including regional location (urban and rural), obese and non-obese.

3.5 Determination of BPA levels in blood samples of few PCOS patients and healthy females

3.5.1 Sample collection and preparation

Among the 100 PCOS patients, randomly 20 females were included in the study for determination of BPA concentration in their blood with the informed consent. Similarly 20 healthy females were involved in the study for the BPA analysis in their blood. BPA was analysed in the leftover blood of the PCOS and healthy females after their hormone analysis. The blood was collected in heparin coated polypropylene vials in order to prevent clotting of the blood and stored in refrigerator until further use. The sample was then carried in thermal bags
while transferring them to the working laboratory for sample preparation for blood BPA analysis. For determination of BPA in the blood samples, 1 ml of blood sample was withdrawn with the help of micropipette into the test tubes. The test tubes prior to use were rinsed thoroughly with distilled water and autoclaved. 20 ml of dichloromethane was added into the test tube containing 1 ml of blood sample. 100 ml of acetonitrile was then mixed in the sample and was allowed for few minutes to form precipitates. After the formation of precipitates, the suspension was centrifuged at 3000 rpm for 10 minutes. The supernatant fluid was separated and concentrated under reduced pressure at 40°C to about 2 ml. The concentrate was then added into the 8 ml of acetonitrile and shifted to a volumetric flask and the volume was adjusted to about 20 ml with distilled water. 5 ml of the above solution was extracted and transferred into the amber coloured glass vials and stored in refrigerator until further use for HPLC analysis with UV detector.

### 3.5.2 HPLC analysis of blood samples

For compound identification of BPA, the standard solution and the external dilutions prepared were analysed using Shimadzu HPLC System equipped with 2475 Pump, C$_{18}$ analytical column (Perkin Elmer, USA; Reverse Phase; 250 mm x 4.6 mm i.d.; 5 µm) and UV detector. Injection volume of 10µl was injected into the C$_{18}$ column. Isocratic elution was carried out with a mixture of acetonitrile: water (v/v) in the ratio of 1:1 at a flow rate of 1ml/min with equilibration time of 10 min. The column temperature was set at 30°C. The UV detector was set at wavelength of 217 nm. The chromatograms were processed using Empower3 software.

The results obtained in the BPA analysis of the blood samples of both the groups were analysed and compared. Further subdivision of the groups was done accounting various factors which included the regions-urban and rural, age group, obese and non-obese among the PCOS patients and healthy females.

### 3.6 Determination of parallelism between the hormone imbalance after exposure to BPA sources and its adverse effects in human health

The data obtained in the above steps was finally compared with the amount of leachate determined in the polycarbonate products, the daily approximate exposure from the polycarbonate and canned BPA sources and the BPA concentration determined in the blood
samples of both the PCOS patients and healthy females. It was determined in the comparative studies the exposure and dependence of the PCOS females in the BPA containing products and the hormonal imbalance along with BPA concentration in their blood samples.