Materials and Methods
3.0 MATERIALS AND METHODS

3.1 Materials

The different materials used for this study are Tuna meat, flexible pouches and ingredients like oil and salt.

3.1.1 Retort pouches

Four types of pouches were used in the study. One was opaque with aluminum foil and the other three were see through pouches which were foil free. Of the three see through pouches one pouch was two layered. The details of all four pouches are given in Table 5. and the photographs in plate 1a-1d.

Table 5. Different types of retort pouches used in the study

<table>
<thead>
<tr>
<th>Details of Pouch manufactures</th>
<th>Description of layers</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Indigenous retort pouch</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. MH Packaging Ltd,</td>
<td>Polyester/Aluminium foil/ cast polypropylene (Opaque)</td>
<td>INOP</td>
</tr>
<tr>
<td>Ahmedabad, Gujarat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Pradeep Lamination, Pune,</td>
<td>Polyester coated with silicon dioxide/ nylon/cast polypropylene (see through)</td>
<td>INST</td>
</tr>
<tr>
<td>Maharasthra</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. MH Packaging Ltd,</td>
<td>Polyester/ cast polypropylene (See through)</td>
<td>INTL</td>
</tr>
<tr>
<td>Ahmedabad, Gujarat</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Imported retort pouch</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. Korean Retort Pouch</td>
<td>Polyester coated with Aluminium oxide /Nylon/ cast polypropylene (See through)</td>
<td>IMST</td>
</tr>
</tbody>
</table>

50
3.1.1.2. Suitability of retort pouch for thermal processing

3.1.1.2.1. Thickness of retort pouches

The total thicknesses of pouches were determined as per ASTM (1964).

3.1.1.2.2. Tensile strength and elongation at break (IS: 2508-1984)

Adequate numbers of strips were cut into suitable size (15 mm x 50 mm). One end of each strip was tightly gripped in the upper clamp after placing the grip loosely in the lower clamp and checking its alignment. The machine was switched on at the pre-adjusted speed (500mm/min). The result of each individual reading to three significant figures in case of tensile strength was recorded. Test strips cut in each principal direction of the paper and tested. The tensile strength at break is calculated in Kg/cm\(^2\) from the original area of cross section. Elongation at break was expressed as % of the original length between the reference lines. The mean values of the 5 results were taken for the calculation.

3.1.1.2.3. Heat seal strength (ASTM-1973)

The strength of the seal was determined by measuring the force required to pull apart the pieces of film, which have been sealed together. The breaking strength of the heat sealed seams was determined in a UTM following ASTM (1.73) F.88-68. The specimen was not more than 25mm in width. The initial jaw separation was 50 ± 1.5mm and the seam equidistant ± 1.5mm between the two jaws. The rate of loading was adjusted so that the lower clamp moves at a rate of 200mm/min. The maximum stress applied to the specimen at yield or breakage was recorded.

3.1.1.2.4. Test for Bursting Strength (Duxbury 1970)

The pouch lips were clamped to the burst strength measuring equipment around the air inlet and between the rubber jaws and tightened well. Air was released gradually for 30 seconds. The pouch can hold air for 25 psi pressure
and 30 seconds without bursting and thus it passes the test. It also showed absence of pinholes in the pouch.

3.1.1.2.5. Bond strength (ASTM-1972)

This was measured by initiating the separation of the layers using diethyl ether or chloroform or toluene and measuring the tensile strength.

3.1.1.2.6. Water vapour transmission rate (IS: 1060 Part II -1960)

The test piece was cut using a template, which was of such a diameter that the edge of the test piece covers half the annular recess of the dish. The dish was filled with desiccant to within 1 to 2 mm of the supporting ring. The test piece was placed on the supporting ring and center. The waxing template was placed centrally over the dish and test piece, and molten wax was run into the annular recess until the wax was in level with the top surface of the template. Air bubbles in the wax was broken with a small gas jet, the wax was allowed to harden. The dish was inspected to ensure that the seal is satisfactory and excess wax on the outside was removed. Filling and sealing of the dish was carried out as rapidly as possible so that the desiccant absorbs a minimum of water vapour from the atmosphere. Care was taken not to damage the test area during the operation or to allow the desiccant to come into contact with it. To facilitate the removal of the template from the wax, thin film of petroleum jelly was applied to the beveled edge before sealing and excess wax removed from the lower surface. WVTR was determined by sealing the open end of the dish containing the desiccant (fused Calcium Chloride) by the test specimen and exposing the dish to the desired RH and temperature conditions. Standard test condition was 37°C and 92% RH, when the desiccant used exerts 2% RH. Increase in weight of the desiccant after a known period of time gives the amount of water vapour transmitted by the specimen. The WVTR of the film is calculated as g/ m² /24 hrs. at 90 ± 2% RH and 37°C.

\[
WVTR = \frac{Q \times 24}{A \times t}
\]
Q - Quantity of water vapour passes through the test material of area ‘A’ sq.meter for ‘t’ hours when the relative humidities on either side maintained at H1 and H2.

Area of test specimen - 50 cm$^2$.

3.1.1. 2.7. Oxygen transmission rate (OTR)

Oxygen permeability of the film was carried out using gas permeability apparatus (Gas and steam permeability, Ats Faar, Societa’ Per Azioni, Milano, Italia) (ASTM, 1982). The test material was cut into suitable size (10 cm dia). B, C and D valve of the instrument was opened and the upper half of the permeability cell was removed. A dried circular filter paper (Whatman No. 1) was placed on the top of the insert after applying vacuum adhesive grease and the sample of film spread over the filter paper. An added mass was placed into the mould. The upper part of the permeability cell was then replaced. All the valves (A, B, C and D) were closed and the vacuum pump was switched on. Then valve C was opened to create the vacuum in the lower portion and it was checked by tilting central vacuum gauge. It should be preferably 0.2 mm Hg. After that the D valve was opened for purging and a valve for removing the atmosphere gas if any. Then the A and D valves were closed. Mercury (Hg) was transferred into the cell by tilting the outer portion and wait for few minutes to attain 0.2 mm Hg vacuum. Valve A was opened and test gas (O$_2$) was applied and the pressure was adjusted using the gas cylinder valve. Then timer was turned on and allowed 15 min for stabilization. Initial vacuum reading was noted from the Eurotherm Chassell. At particular interval vacuum was noted and the gas transmission rate was calculated and expressed as mL m$^{-2}$ 24 h$^{-1}$ at 1 atm. pressure at 24°C.

3.1.1. 2.8. Residual air test (Shappee et al., 1972)

The test was performed by holding the pouch inverted below water under a funnel attached to a graduated cylinder filled with water. A corner of the pouch was cut open under the funnel and the air is squeezed out. The amount of residual air in the pouch was measured as the water displacement in the cylinder.
The volumetric measurements of air were corrected to atmospheric pressure by Boyle's law:

\[ V_1 = \frac{(P_a - W_h) V_m}{P_a} \]

Where, \( V_1 \) = residual air in pouch at atmospheric pressure (m L)
\( P_a \) = atmospheric pressure (inches of mercury)
\( V_m \) = volume of measured air (m L)
\( W_h \) = pressure of water in graduated cylinder (inches of mercury)

Where, \( W_h = \rho gh \)

where, \( \rho \) = density of water (kg m\(^{-3}\))
\( g \) = acceleration due to gravity (m s\(^{-2}\) or N kg\(^{-1}\))
\( h \) = height of water in graduated cylinder (m)

3.1.1.2.9. Overall migration test (IS: 9845-1998)

Overall migration test was performed by using the food stimulants such as distilled water, and n-heptane. The pouch was filled to capacity with pre-heated stimulant at test temperature and closed. The pouches were exposed to specified temperature and maintained for the specified duration of time (121°C for distilled water and 3% acetic acid and 66°C for n-heptane). After exposure for the specified duration, the pouch was removed and the extractant was quickly transferred into clean glass beaker with three washing with stimulant. The extractant was evaporated to about 50-60 ml and transferred into a clean tarred stainless steel dish along with three washings and further evaporated to dryness in an oven at 100°C. The dishes were cooled in a desiccator for 30 minutes and weighed. The extractives were expressed in mg/dm\(^2\).

3.1.1.2.10. Laminate for product resistance (Gopakumar, 1993)

Two sets of pouches were taken for control and sample. 6 pouches were filled with the product to be packed and other 6 with water (control). All the pouches were sealed. The samples and control were retorted in a pressure retort.
suitable for retort pouch processing for 45 and 30 min. for sample and water filled pouches respectively. After processing pouches were cooled to ambient temperature, contents were emptied and washed thoroughly with cold water. The pouches were cut into strips of 1 x 25 mm size from the machine direction and another pouch in transverse direction, cutting across the seam area. The seam was pulled apart and delaminated plies were examined. The laminate plies can be separated apart using Universal Testing Machine and observed the bond strength in g/25mm.

3.1.1.2.11. Process resistance of pouches (Gopakumar, 1993)

One crumpled and other uncrumpled pouches were placed in a retort containing some water and heated to 121° C (15 psi steam pressure). After 30 minutes pouches were cooled and taken out and examined carefully for delamination.

3.1.2. Fishes

Fishes used for the study were yellow fin tuna (Thunnus albacares) (Plate 4.) Fishes were obtained from the Cochin fishing harbour, Thoppumpady, Cochin. Fishes were purchased according to the requirement and brought to the laboratory in iced condition. The tuna was washed, bled in chilled water and loined. The red meat was removed from the loins and then the loins were cut into steaks of 1.5 mm thickness (Plates 5 and 6). The steaks were then brined in 5 % brine solution (w/v.) for 1 h. The steaks were then drained and used for the smoking.

3.1.3. Oil

Double refined ground oil was used for filling the pouches for smoked tuna in oil medium.

3.1.4. Salt

Salt of edible quality confirming to IS: 594-1962 was used for pretreatment before smoking and for preparation of the filling medium for smoked tuna in brine.
3.2. Machineries and accessories

3.2.1. Kerres Smoke Kiln

The Smoke kiln, Kerres of Germany (Model No.CS 350 'G' EL) is a stainless unit which has an open chamber. The chamber contains provisions for placing detachable trays on which the fish to be smoked is kept. It has a heating element and fan at the top for temperature control and spreading the smoke in the chamber. There is an inlet for smoke generation below the chamber and an outlet at the top where the smoke escapes out after passing over the fish. Digital controls are provided for setting the temperature and time. Smoke generation is done manually in the chamber below the kiln and the smoke is allowed to pass upwards through an opening in the chamber. The quantity of smoke was controlled by adjusting the inlet valve. There is an inlet to continuously feed the wood once the smoking has commenced. The photograph of the smoke kiln is given in Plate 2.

3.2.2. Pilot scale retorting unit

The pilot scale mill wall model 24 rotary retorting system (John Fraser and Sons Ltd, FWS House, Stoddard Street, New Castle-upon-Tyne, UK) was used for the experiments (Plate 3.). This pilot scale retorting system performs laboratory scale thermal processing in a manner which ensures close simulation with commercial scale equipment and which produces a high degree of process reproducibility and accuracy. This system comprises three major components; the retort, the receiver and the control system. The retort provides a chamber in which the product is subjected to the required thermal process. The receiver provides a pressure to balance the overpressure in the retort during super heated water cooks and during overpressure cooling. The control system provides the means to sequence process events, regulate energy flows and document retort temperature and pressure.

Retort is constructed of mild steel which can withstand a working pressure of 3.5 bars having a dimension of 594 mm inside diameter X 650 mm inside length on parallel portion. It has a standard square cage, which is perforated with
Plate - 2 Smoke Kiln

Plate - 3 Overpressure Autoclave
side slots. The speed of rotation of the cage ranged from 0 to 51 rpm and was electronically controlled. Instrument pockets are provided on the right side of the shell. These include pressure gauge, retort thermometer, pockets for thermocouple glands and petcock at the rear end. A water gauge is provided on the right hand side of the retort to know the water level inside the retort. A pressure release valve is provided on the retort to release the pressure if it is above 55 psig. The pressure gauge, which is provided on the retort, has got a range of 0 to 60 psig. A 4-blade stainless steel fan is fitted to the retort to create considerable turbulence within the retort during processing to ensure well mixing of steam and that no stagnant air pockets were allowed to exist. The retort is connected to a very efficient cooling system. As soon as the process is over, steam can be switched off and water can be allowed to enter into the retort with the help of a water pump from the water-storing tank. This will provide a very efficient cooling mechanism by spraying water from the top of the retort. The same water can be recirculated with the help of a recirculating pump (Myson MSK 50 – 2/2090).

The receiver is also constructed of mild steel and has got a working pressure of 50 psig having a dimension of the receiver is 594 mm inside diameter x 850 mm on parallel side. It has got a water gauge with the gauge top which indicates the receiver is full and the gauge bottom which indicates receiver below overflow level. It has got a pressure release valve and the setting is on 55 psig. It has got a pressure gauge with a range of 0-60 psig. The pressure in the receiver is hydraulically and pneumatically transmitted to the retort at the points in the sequence when the retort is required to be at over pressures. Two modulating valves control the receiver pressure; one regulates air into the vessel and the other acts on the vent and regulates air out. The controller is designed with a dual output to operate the system. The pressure control valve is connected to the vent valves on the receiver. The transfer line between the two vessels must be open when the pressure is controlled with the sensor mounted on the retort.

The control systems has a Programmable Logic Controller (PLC) assisted manual controls i.e. retort operation performed manually but with the help of discrete electronic programmable input detector controllers for temperature and
pressure. The control system has got a digital temperature indicator and pressure indicator. A digital three-pin circular chart recorder is fitted to record retort temperature and pressure and receiver pressure. A eurotherm digital indicator is fitted to display cage rotation speed. The instrument is connected to the 0-10 V output of the motor control unit and is scaled for 0-51 rpm. A digital electronic timer is provided to assist the timing of the cook period. The timer is integrated into the PLC (Mitsubishi F1 series 60 I/O) monitor system and is used to prompt the operator to begin cooling. The PLC system is provided to monitor system safety. It observes retort door interlocks and temperature and pressure alarms and acts upon the automatic valves pump and cage drive (Plate 3).

3.2.3. Packing glands and accessories

Ellab GKM-13009-CXXX packing glands for all kinds of containers were used for the experiments. The GKM is as standard delivered with a GKM-U rubber O ring. For special applications it can be used with wedge washers and silicon washers. Packing glands are usually made up of brass, stainless steel or polyoxymethylene.

3.2.4. Standard thermocouple probes

The probes used for the experiments are that of Ellab Type SSE- G700-SF (ELLAB Co. Denmark) stainless steel electrode with a length of 100 mm and diameter of 1.2 mm. These probes are copper/cupronickel thermocouples; they are sealed probes with the conductor being insulated from the process medium. The pouches are E.M.F. characteristics corresponding to the probe output voltage of Cu/Cu Ni thermocouples.

3.2.5. Ellab CTF 9008 Precision Thermometer and Fo- value computer

Temperature range of the instrument is −100.0 to +350.0°C. Resolution of the instrument is 0.1°C. There are 8 channels with selective functions for product (Tc) and chamber (Ta) temperatures. These 8 channels are updated within 4 seconds with each channel getting updated within 30 seconds. The Fo constants are programmed T=121.1°C, Z=10°C and Cook value constants T=100°C,
Z=33°C. The print out interval from the instrument can be selected and it varies from 30 seconds to 60 minutes. The print out shows Tc and Ta min/max, peak temperatures, channel numbers and the corresponding Fo and cook value of each channel.

3.2.6. Vacuum sealing machine

The vacuum sealing machine (Model QS 400 VD) supplied by M/s Sevana Electrical Appliance Pvt. Ltd., Box No. 2, Kizhakkambalam, Kerala, India, was used for sealing the pouches (Plate 12).

3.2.7. Food Texture Analyzer

It is a general-purpose material-testing machine manufactured by Lloyd instruments, UK (Model LRX plus). The software used in the instrument is Nexygen which gives data output to a computer and printer. The main part of the instrument was fitted with a load cell of 50 N. The LRX plus machine was fitted with two magnetically activating limit stops to stop the machine. The speed of the cross edge movement varies from .01-1016 mm/min. The unit has a liquid crystal display (LCD) to show set up information, load and extension values and a key pad to input information for operating the machine when under the control of the console. The operating status of the machine was shown and described on the display. The display, which has 4 lines of forty characters, was used to show or request information. The lower lines are split into four blocks, one block above each soft key to indicate the function of the key.

3.2.8. Hunter lab MiniScan® XE plus

The Hunter Lab MiniScan® XP Plus spectro colorimeter, model No D/8-S (Hunter Associates Laboratory Inc., Reston, VA, USA) with geometry of diffuse 80 (Sphere-8 mm view) and an illuminant of D 65 optical sensor and 100 standard observer was used for instrumental colour measurement of samples. The colour values are expressed using the standard CIE L*a*b* system. L*, a*, and b* values (non dimensional units) refer to the three axes of the system: a lightness axis (white (100)- black (0); L*) and two axes representing hue and chroma, (a*) one
red (positive)-green (negative) and the other ($b^*$) blue-yellow. This system provides an unambiguous description of color and has the advantage that color differences between samples can be determined using simple computer programs.

3.2.9. Spectrophotometer

Spectrophotometer of Spectronic 20 Genesys model manufactured by Thermo Spectronic, Rochester, NY 14625 was used for the study.

3.3. Methods

3.3.1. Brining of tuna

The tuna steaks were immersed in 5% salt solution in the ratio 1:1 (wt: v) for 1 hour and drained (Plate 7). The drained tuna steaks were packed in laminated covers made of polyester / polythene, sealed and kept overnight in the chilled condition ($2 \pm 1^\circ C$) for equilibration of salt content.

3.3.2. Standardization of smoking parameters

The salted tuna steaks were wiped off excess moisture and surface dried on trays in a smoke kiln at 45 °C for 30 min. Smoke generation was done manually using saw dust from teak (*Tectona grandis*), Cheruteak (*Callicarpa tomentosa*), Cashew (*Anacardium occidentale*), Kolamavu (*Buchania axillaries*), Acacia (*Acacia auriculiformis*), Maruthu (*Terminalia paniculata*), Anjily (*Artocarpus hirsuta*) and husks from coconut (*Cocos nucifera*). The saw dust from these woods and coconut husks were ignited in different batches and smoke arising from them were allowed to flow over the tuna steaks. The process of smoking is given in Plates 8 and 9 and smoked tuna in Plate 10. The tuna was smoked to different durations of 30, 60 and 90 minutes at 75°C. The quantity of smoke allowed was controlled by adjusting the valve. The steaks were turned over after regular interval to get a uniform colour. After smoking the steaks were cooled and removed from the trays. The selection of wood and duration of smoking was mainly based on the organoleptic characteristics like flavour and colour and the benzo (a) pyrene content of the smoked fish. For preparation of smoked and thermal processed tuna in the three different forms,
Plate - 4 Yellowfin Tuna

Plate - 5 Tuna Loins

Plate - 6 Tuna Steak
Plate - 7 Brining of Tuna

Plate- 8 Tuna Loaded into Smoke Kiln

Plate - 9 Smoked Tuna in Kiln
Plate - 13 Loading into Autoclave

Plate - 14 Smoked and Thermal Processed Tuna in Pouches
Plate - 15 Smoked and Thermal Processed Tuna Drypack

Plate - 16 Smoked and Thermal Processed Tuna in Brine

Plate - 17 Smoked and Thermal Processed Tuna in Oil
smoking was done for one hour at 75°C using dried coconut husks, which had a moisture content of about 20-25%.

3.3.3. Thermal processing of smoked tuna in brine

Smoked tuna steaks were packed into the four different retortable pouches used in the study. The filling ratio was 60 g fish and 40 ml liquid medium. Hot brine solution (1% salt) was added as the filling medium (Plate 11). The pouches were then sealed in a liquid vacuum packaging machine (Plate 12) and loaded onto trays for further processing in the retort. The retort was loaded to the full capacity. Approximately 50 pouches were loaded in each batch. Pouches were heat processed to a Fo value of 10 min at 121.1°C in a stationary retort (Plate 13). Heat penetration characteristics were recorded using thermocouples connected to Fo value cum cook value recorder. At the end of the process the pouches were cooled immediately. The pouches were then dried, labeled and stored (Plate 14 and 16). Three pouches each were kept at 37°C for 15 days and at 55°C for 5 days for determination of sterility.

3.3.4. Thermal processing of smoked tuna in oil

Smoked tuna steaks were packed into retort pouches with a filling ratio of 60 g fish and 40 ml sunflower oil. The oil was heated to 90°C and filled into the pouches. All the four pouches were then sealed using a vacuum packaging machine for liquids and loaded on to trays for further processing in the retort (Plate 15). The rest of the operations were similar to that of tuna in brine.

3.3.5. Thermal processing of smoked tuna dry pack

Smoked tuna steaks (100g) were packed into the four different retortable pouches used in the study. The pouches were then sealed in a vacuum packaging machine and loaded on to trays for further processing in the retort (Plate 17). The rest of the operations were similar to that of tuna in brine.
Flow Chart - 1. Pouch packing of Smoked Tuna in Oil

1. Raw material (Stored at 1-4°C)
   - Washing (Chilled water)
   - Beheading, Gutting and Removing fins
   - Bleeding and Washing (Chilled water)
   - Preparation of steaks from loins
   - Cold blanching in 5% brine solution for 60 minutes
   - Surface drying for 30 minutes at 45°C
   - Smoking at 75°C for 1 hour

2. 60 g fish
   - Filling into retort pouches
   - Vacuum sealing
   - Retorting (at 121.1°C)
   - Cooling and washing
   - Labelling & Storage

3. 40 ml hot refined oil
Flow Chart - 2. Pouch packing of Smoked Tuna in Brine

1. Raw material (Stored at 1-4°C)
2. Washing (Chilled water)
3. Beheading, Gutting and Removing fins
4. Bleeding and Washing (Chilled water)
5. Preparation of steaks from loins
6. Cold blanching in 5% brine solution for 1 hour
7. Surface drying for 30 minutes at 45°C
8. Smoking at 75°C for 1 hour
9. Filling into retort pouches
10. Vacuum sealing
11. Retorting (at 121.1°C)
12. Cooling and washing
13. Labelling & Storage (28 ± 2°C & 37 ± 2°C)
Flow Chart - 3. Pouch packing of Smoked Tuna as dry pack

1. Raw material (Stored at 1-4°C)
2. Washing (Chilled water)
3. Beheading, Gutting, Removing fins
4. Bleeding and Washing (Chilled water)
5. Preparation of steaks from loins
6. Cold blanching in 5% brine solution for 1 hour
7. Surface drying for 30 minutes at 40°C
8. Smoking at 75°C for 1 hour
9. Filling into retort pouches (100 g)
10. Vacuum sealing
11. Retorting (at 121.1°C)
12. Cooling and washing
13. Labelling & Storage (28 ± 2°C & 37 ± 2°C)
3.3.6. Heat penetration and thermal process evaluation

The thermal data were taken by inserting thermocouple needles into the product. Thermocouple output was measured by using an Ellab CTF 9008 data recorder. Time-temperature data were taken at an interval of one minute. The heat penetration data were plotted on a semi log paper with temperature deficit (RT-CT) on log scale against time. Lag factor for heating (Jh), slope of the heating curve (fh), time in minutes for sterilization at retort temperature (U) and lag factor for cooling (Jc) were determined. The process time was calculated by mathematical method (Stumbo, 1973). The graph for Fo value, cook value, retort temperature and product temperature were drawn from the time- temperature data. Actual process time is determined by adding process time (B) and the effective heating period during come up time i.e. 58% of the come up time.

3.3.7. Quality analysis of retort pouch products

Tuna in oil, tuna in brine and tuna as dry pack heat processed to Fo 10 in four different pouches were stored at ambient temperature 28 ± 2°C and also at an accelerated temperature of 37± 2°C to determine the shelf life. Triplicate samples were periodically analysed once in a month for determining changes in thiobarbituric acid (TBA) value, free fatty acid (FFA), pH, changes in texture profile by instrument methods, changes in CIE L*a*b* colour values and organoleptic sensory methods for determining the overall acceptability.

3.3.7.1. Determination of pH (IS: 2168-1971)

About 5 g of the sample was homogenized with 10 ml distilled water and the pH was recorded using a digital pH meter.

3.3.7.2. Determination of moisture (AOAC, 2000)

A known weight of homogenized sample (10g) was weighed in a preweighed clean petridish on an electronic balance. The samples were allowed to dry by placing in a hot air oven at 105°C for 16 h, then cooled in a desiccator.
and weighed until constant weight was obtained. The moisture content was calculated and expressed as percentage.

\[ \% \text{ Moisture} = \frac{\text{Loss in weight} \times 100}{\text{Weight of the sample}} \]

3.3.7.3. Determination of crude protein (A.O.A.C, 2000)

About 0.5-1g of the minced sample was transferred into a Kjeldahl flask of 100 ml capacity. A few glass beads and a pinch of digestion mixture (8 parts K\(_2\)SO\(_4\) and 1 part CuSO\(_4\)) and 10 ml of concentrated sulphuric acid were added. It was digested over a burner until the solution turned colourless. To the digested and cooled solution, distilled water was added in small quantities with intermittent shaking and cooling until the addition of water generated no heat. It was transferred quantitatively into a 100 ml standard flask and made up to the volume. With a 2 ml pipette made up solution was transferred to the reaction chamber of the Micro-Kjeldahl distillation apparatus. 2 drops of phenolphthalein indicator and 40% sodium hydroxide were added till the indicator changed to pink. Distillation was done for 4 minutes and ammonia liberated was absorbed into 2% boric acid containing a drop of Tashiro’s indicator. The amount of ammonia liberated was determined by titration with 0.01 N standard sulphuric acid. Crude protein was calculated by multiplying total nitrogen content with conversion factor of 6.25 and expressed as percentage

\[ \% \text{ Crude protein} = \text{nitrogen content} \times 6.25 \]

3.3.7.4. Estimation of crude fat (AOAC, 2000)

About 2-3 g of accurately weighed moisture free sample was taken in a thimble plugged with cotton and extracted with petroleum ether (40-60\(^\circ\)C boiling point) in a soxhlet apparatus for about 10 h at a condensation rate of 5-6 drops per second. Excess solvent was evaporated and the fat was dried at 100\(^\circ\)C to constant weight. The crude fat was calculated and expressed as percentage.

\[ \% \text{ Crude fat} = \frac{\text{Weight of fat} \times 100}{\text{Weight of the sample}} \]
3.3.7.5. Determination of ash content (AOAC, 2000)

About 1-2 g of the sample was transferred into a preweighed silica crucible. The samples were then charred by placing in a muffle furnace at 550°C for 4 hrs until a white ash was obtained. Crucibles were weighed after cooling in a desiccator and percentage of ash was calculated.

\[
\text{% Ash} = \frac{\text{Weight of residue} \times 100}{\text{Weight of the sample}}
\]

3.3.7.6. Determination of Thiobarbituric acid (TBA) value (Tarladgis et al., 1960)

About 10 g of the sample were macerated with 100 ml 0.2 N HCl and made into a slurry. Slurry was poured to a round bottom flask and connected to a TBA apparatus. The macerated sample was distilled by steam distillation method and 50 ml of the sample was collected in 10 minutes. Accurately weighed 0.288 gms of TBA standard was dissolved in 100ml glacial acetic acid in hot water bath and cooled to room temperature. Five ml of the samples were taken in test tubes and 5ml of the prepared TBA reagent was also added. A blank was also made with distilled water. Then the samples were kept in boiling water bath for 30 minutes for colour development. The developed colour was read at 538 nm wavelengths against blank in a spectrophotometer. The TBA value is expressed as mg malonaldehyde / kg of fish.

3.3.7.7. Determination of Free Fatty Acids (AOCS, 1989)

About 10 g of the sample was blended with anhydrous Na₂SO₄ in a mortar. The blend was shaken with chloroform and kept under dark overnight and filtered. 20 ml of the extract was taken in to a clean beaker. Chloroform was evaporated on a water bath and weight of fat was determined. Another 20 ml of extract was transferred in to a conical flask. Chloroform was evaporated off. To this 10 ml of neutral alcohol was added and warmed. It was titrated against 0.01 N NaOH using phenolphthalein as indicator. Percentage of free fatty acid (FFA) was calculated as oleic acid.
3.3.7.8. Total Volatile Base Nitrogen (Conway, 1950)

Total volatile base in the sample was determined as total volatile base nitrogen (TVB-N) by the micro diffusion method. One ml of standard N/100 sulphuric acid was taken in the inner chamber of the diffusion unit. To the outer chamber 1 ml of TCA extract was added followed by 1 ml of saturated potassium carbonate. The unit was then sealed with the glass lid and kept undisturbed overnight. The amount of un reacted acid in the inner chamber was determined by titrating against standard N/200 sodium hydroxide with 2 drops of Tashiro's indicator. Similarly a blank was also run. Total volatile base nitrogen (TVB-N) was calculated and expressed in mg N/100g of the sample.

3.3.7.9. Total Amino acids (Ishida et al., 1981)

Principle

The amount of each amino acid present within a given protein does not vary from molecule and can provide useful information about the nature of the protein molecule. In a typical analysis of the amino acid content of a protein, peptide bonds are broken by acid hydrolysis with 6N HCl at 110°C (24h) so that the released amino acids can be assayed. The amino acid tryptophan is not stable to acid digestion in the presence of even trace amounts of oxygen and is estimated separately by alkali digestion.

Sample preparation

About 100-150 mg of sample was weighed accurately into a heat sealable test tube. 10 ml of 6 N HCl was added and the tube was heat sealed after filling pure nitrogen gas. Hydrolysis was carried out in an hot air oven at 110°C for 24 hours. After the hydrolysis, the contents were removed quantitatively and filtered into a round bottom flask through Whatman filter paper No.42. The contents of the flask were flash evaporated to remove traces of HCl and the process repeated for 2-3 times with added distilled water. The residue was made up to 10 ml with 'C' buffer (sodium citrate tribasic, perchloric acid, n- caprylic acid, pH 2.2).
HPLC Analysis - The sample was prepared and filtered again through a membrane filter of 0.45 μm and 30 μl of this was injected to Shimadzu HPLC-LC10AS consisting of column packed with a strongly acidic cat-ion exchange resin i.e. styrene di-vinyl benzene copolymer with sulfonic group. The column is Na type i.e. ISC-07/S1504 Na with a length of 19 cm and 5 mm diameter. The mobile phase of the system consisted of two buffers, Buffer A (Tri sodiumcitrate-32.7g, Methanol of 140 ml, Perchloric acid 16.6 ml pH 3.2, make up to 2 liter) and buffer B (Tri sodium citrate 117.6 g, Boric acid 24.8 g, 4N NaOH 45 ml, pH 10). The oven temperature was maintained at 60° C. The amino acids were eluted from the column by stepwise elution i.e. acidic amino acids first followed by neutral and then basic amino acids. The amino acid analysis was done with non-switching flow method and fluorescence detection after post-column derivatization with O-phthalaldehyde. In the case of proline and hydroxy proline, imino group was converted to amino group with sodium hypochlorite. Amino acid standards (Sigma Chemical Co., St. Louis, USA) were also run to calculate the concentration of amino acids in the sample. Calibration of equipment using standards was done before the start of analysis.

Quantification of amino acids: The standard and the sample were analyzed under identical conditions. The elution time of the amino acids of the sample was compared and identified with those of the standard. Quantification of amino acid was done by comparing the respective peak areas in the chromatogram of the sample and the standard. The amino acid content was calculated as follows,

\[
\text{mg amino acid/g tissue} = \frac{\mu\text{mol}*\text{mol.wt}*\text{volume made up}*1000*100}{1000*1000*20*\text{wt. of sample}}
\]

The amount of each amino acid is expressed as mg amino acid/g tissue

3.3.7.10. Estimation of Tryptophan (Sastry and Tummuru, 1985)

About 200-250 mg of sample was hydrolysed with 10 ml of 5% NaOH at 110° C for 24 hours in a sealed tube filled with pure nitrogen. The hydrolysate was neutralized to pH 7.0 with 6 N HCl using phenolphthalein indicator and checked with BDH pH paper. The volume was made up to 100 ml with distilled water. This was then filtered through whatman filter paper No.1 and filtrate was used for estimation. 0.1 ml of 2.5% sucrose and 0.1 ml of 0.6% thioglycolic acid
were added to test tube containing 4 ml of 50% H₂SO₄ and kept for 5 min in water bath at 45-50° C and cooled. An aliquot of the sample was then added to the test tubes. The experiment was repeated with 0.1 to 0.8 ml of standard tryptophan (10 μg/ml). The volume was made up to 5 ml with 0.1 N HCl and allowed to stand for 5 minutes for the development of colour. The absorbance was measured against a reagent blank at 500 nm.

3.3.7.11. Determination of biogenic amines (Ozogul et al., 2002)

The biogenic amines content in fish was determined in HPLC by pre-column derivatisation with benzoyl chloride as described by Redmond and Tseng, (1979) with modification in gradient elution system as per the method of Ozogul et al., (2002) using acetonitrile and water. The gradient system and the flow rate were modified depending on the retention time of the standard amine solution to get good resolution within a short time. The standard amine solution was prepared so as to give a 10mg free base each amine per ml.

**Derivatisation of standard amine solution with benzoyl chloride**

The benzoyl derivative of the Biogenic amines were done by following the Schotten- Baumann benzoylation reaction under alkaline condition as described by Redmond & Tseng, (1979) but with little modification of the sample. For enhancing the reaction of amines, 2% benzoyl chloride in acetonitrile was used.

For derivatisation of standard amine solution, 50 micro litre of standard amine solution (10mg/ml) was added with 1ml of 2M NaOH followed by addition of 1ml of 2% benzoyl chloride (in acetonitrile). Then it was mixed thoroughly in a vortex mixture for 1 minute. The reaction mixture was left at room temperature (25°C) for 30 minutes to complete the benzoylation reaction. The reaction was stopped by the addition of 2ml of saturated NaCl solution. After that it was extracted with 4ml of di-ethyl ether by centrifugation at 3000 rpm for 5 minutes. Thereafter the upper organic layer was transferred to a clean tube and evaporated to dryness in a stream of nitrogen. The residue was dissolved in 0.5ml of acetonitrile and 20 μl aliquots were injected for HPLC analysis.

Preparation of sample was done as per the method of (Yen & Hseih, 1991). 10 g of the sample was homogenized with 6% TCA and filtered through
Whatman No.1 filter paper. The filtrate was made up to 100 ml with TCA. 2ml of this extract was derivatised with benzoyl chloride by the same procedure as described in derivatisation of standard amine solution.

**Chromatographic condition**

Chromatographic separation was done by continuous gradient elution with acetonitrile (solvent A) and HPLC grade Millipore water (solvent B) as described in Ozogul et al., (2002). A gradient started with 50% acetonitrile and increased to 80% in the 6th minute. The pressure was maintained between 48-52 Pascal throughout the separation period. Total separation of seven amines was completed within 9 minutes. Detection was done using Photo Diode Array (PDA) detector.

For calibration curve, 5 standard concentrations of amines mixtures were prepared and injected in a series comprising 10mg/ml, 5mg/ml, 1mg/ml, 0.1mg/ml and 0.01mg/ml standard concentration. The standard curve was prepared corresponding to Hitachi-Merck HPLC System Manager Software. 20 µl sample was injected for analysis.

**3.3.7.12. Estimation of Polyaromatic hydrocarbons (Granby and Spliid,1995)**

Principle: PAHs, a group of lipophilic compounds are extracted from fish with n-hexane after saponification. The two fractions are eluted in a column of alumina and silica by normal hexane to remove low molecular weight hydrocarbons. On further elution with 1:1 dichloromethane and normal hexane gives high molecular weight hydrocarbons, one of which is benzopyrene, this portion is then dried and taken in mobile phase, acetonitrile and analysed in HPLC.

The method of estimation of PAH compounds was done as per the method of Granby and Spliid (1995) with some modifications. Approximately 10 g of meat homogenate was saponified by refluxing for 2 h with 10 ml of a 4 N KOH solution and 40 ml of ethanol. Then 40 ml of the 4 N KOH solutions were added and the mixture was allowed to stay overnight. Afterward, the sample was transferred to a separatory funnel and extracted with 3 times with 25 ml of n-hexane. Organic phases were dried by filtering through a funnel with anhydrous sodium sulfate and
vacuum evaporated to approximately 1 ml by n-hexane. The chromatographic clean-up was performed on a column (20 cm length, 1 cm internal diameter) filled with 4 g aluminium oxide above 4 g silica gel that had been activated overnight at 250°C and 120°C, respectively. The sample was added to the top of the column and eluted with 25 ml of n-hexane (fraction containing paraffin hydrocarbons) and 30 ml of n-hexane/dichloromethane (1/1) (fraction containing polyaromatic hydrocarbons). This fraction was evaporated to dryness by vacuum and nitrogen flow and dissolved in 1 ml of acetonitrile. The detection of PAHs was performed on a Shimadzu high performance liquid chromatography equipped with photo diode array detector set at 254 nm. The LC column is a Lichrospher –PAH, RP 18. HPLC is conditioned by passing mobile phase, HPCL grade acetonitrile, 95% in deionised water. Sixteen different PAH compounds were detected. Recoveries were determined from an external standard PAH mixture Supelco (48743) at spiked levels of 200 µg /100 ml wet weight.

\[
\mu g \text{ PAH/g meat} = \frac{\mu g \text{ PAH in sample injected} \times \text{vol. of sample (1 ml)} \times 2}{\text{Extract vol. (20 µl)} \times \text{weight of sample}}
\]

3.3.7.13. Estimation of total carbonyls. (Henick et al., 1958)

Preparation of Carbonyl Free Benzene: To 1 litre of benzene 5g of 2, 4 dinitro phenyl hydrazine (DNPH) & 1g of trichloroacetic acid were added. The contents were refluxed for an hour & distilled in all glass apparatus. The first 50 ml of the distillate was discarded.

Preparation of Carbonyl Free alcohol: To 1 litre of alcohol about 7g of Aluminium dust & 10g of potassium hydroxide pellets were added. The mixture was refluxed for 1 hour & distilled in an all glass apparatus discarding the first 50ml of distillate.

Five g of meat was made moisture free and extracted in carbonyl free benzene to 50ml in a volumetric flask. Five ml of this pipette out into a 50 ml volumetric flask and to it 3 ml of 4.3% tri-chloroacetic acid (4.3 % in benzene) and 5 ml of 2,4-dinitro phenyl Hydrazine (0.05% in benzene) was added and incubated at 60° C for half an hour to convert free carbonyl into hydrazine. After cooling 10 ml of potassium hydroxide pellets (4% in ethyl alcohol) was added and
the volume was made up to 50 ml with ethanol. After 10 minutes, absorbency was measured at 480 nm using a spectrophotometer. A blank was prepared in the same manner substituting sample extract, instead with 5ml benzene. A standard curve was drawn using Valeraldehyde (50-250 µg) in 5ml of benzene instead of sample extract. The total carbonyl was calculated with the help of standard curve & expressed as mg of Valeraldehyde per 100g of sample.

\[
\text{Conc. in } \mu g \times \text{First vol. made up (50 ml)} \times \text{Final Vol. Made up (50 ml)} \times 100 \div 1000 \times \text{weight of sample in mg} \times 5
\]

3.3.7.14. Estimation of total phenols (AOAC, 2000) Phenols (5530) / 5-43

Steam distillable phenols were estimated by the direct photometric method. Steam distillable phenolic compounds react with 4- aminoantipyrine at pH 7.9± 0.1 in the presence of potassium ferricyanide to form a colored antipyrine dye. This dye is kept in aqueous solution and the absorbance is measured at 500 nm.

10 g sample is mascerated thoroughly with 50 ml distilled water and transferred into a distillation flask and indirectly steam distilled. 100 ml of the distillate is collected in a 250 ml beaker. To this 2.5 ml 0.5 N ammonium hydroxide solution is added and then the pH is immediately adjusted to 7.9 ± 0.1 with phosphate buffer. To this 1 ml 4 - amino antipyrine solution is added and mixed well. Then 1 ml of potassium ferricyanide solution is added and mixed well. 100 ml distilled water and a series of 100 ml phenol standards containing 0.1, 0.2, 0.3, 0.4, and 0.5 flask 100 mL distillate, or a portion containing not more than 0.5 mg is prepared.

\[
\text{Mg phenol/kg} = \frac{C \times D \times 1000}{E \times B}
\]

Where \(B\) = mg of original sample, \(C\)=mg standard phenol solution, \(D\)= absorbance of sample, \(E\)= absorbance of standard phenol solution.
3.3.7.15. Determination of Texture Profile

The TPA method of Bourne (1978) based on compression of samples with Universal Testing Machine (Lloyd instruments LRX plus,) was used to objectively evaluate textural differences between treatments. Uniform size tuna samples from pouches were used for the analysis. The load cell used was a cylindrical probe of 50mm diameter with 50 N capacity. The samples were compressed twice to 40% of their original height; at a crosshead speed of 12 mm/min. Force by time data from each test were used to calculate mean values for the TPA parameters. The values for hardness 1 and 2 (the resistance at maximum compression during the 1st and 2nd compression), cohesiveness (ratio of the positive force area during the 2nd compression to that during the 1st compression of Area 2/Area 1), springiness (ratio of the time duration of force input during the 2nd compression to that during the 1st compression of length 2/length 1), and chewiness (hardness1 x cohesiveness x springiness in kg mm) were determined as described by Bourne (1978). At least five duplicates were done and average readings were taken.

3.3.7.16. Sensory Test (IS: 6273[II]-1971)

Sensory evaluation was based on characterization and differentiation of the various sensory characters such as appearance, colour, texture, odour and flavour. Score was given based on a ten-point scale by trained taste panel members (Annexure 1), as per guideline given by IS: 6273[II]-1971. Scores 9-10, 6-8, 4-5 and 1-3 were taken for excellent, good, fair and poor respectively for each of the sensory characteristic. The 10 point was given for prime quality product, whereas 4 was the limit for unacceptability after which the product was rejected. The characteristics covered under the taste panel were appearance, colour, odour, flavor, texture and overall acceptability. Texture characteristics such as succulence, toughness, fibrosity, firmness and chewiness were also studied.
3.3.8. Commercial sterility test (IS: 2168-1971)

The thermally processed samples were incubated at 37°C for 15 days and 55°C for minimum of 5 days. The incubated pouches were aseptically opened and 1-2g of the samples were taken by a sterilized forceps and inoculated into the sterilized fluid thioglycolate broth in test tubes. Little sterilized liquid paraffin was put on to the top of the broth to create anaerobic condition and incubated at 37°C and at 55°C.

3.3.9. Statistical analysis (SPSS, 2000)

The SPSS 10.00 (SPSS, 2000) statistical packaging was used for analysis of the experimental results. Sufficient numbers of samples were carried out for each analysis. Results were expressed as mean ± standard deviation. Analysis of variance was used to calculate significant difference (p<0.05) between different pouches and samples stored at ambient temperature and accelerated temperature.
Annexure 1:
SENSORY EVALUATION OF THERMAL PROCESSED SMOKED TUNA
Assessor: .......................................................... Date: ..................

(Please score the sample characteristics by placing the relevant score)

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<td>iii. Succulence</td>
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<td>v. Chewiness</td>
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<td>Overall acceptability</td>
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(Please score the sample characteristics according to the following scale)

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Signature