MATERIALS & METHODS
3. MATERIALS AND METHODS

3.1. Preparation of Maillard reaction products (MRPs)

Amino acids like glycine, methionine, tryptophan, aspartic acid and lysine were procured from sigma chemical co. USA, and glucose from BDH Company.

MRPs were prepared by refluxing 30 and 60 mM concentrations of glucose in the presence of similar concentrations of glycine, methionine, tryptophan, aspartic acid and lysine in 100 ml of water for 1 and 2h over a sand bath maintained at 100 to 110° C. Losses in water content were periodically restored for maintaining the final volume. Four sets of each combination of 30 mM/1h, 30 mM/2h, 60 mM/1h and 60 mM/2h (of glucose and amino acids) were prepared for storage studies at 5° C with a methyl linoleate model system.

3.2. Preparation of methyl linoleate model system

Chloroform solution (25ml) containing a weighed amounts of (250mg) methyl linoleate, was mixed with 5g microcrystalline cellulose in a 250 ml capacity RB flask. Chloroform was removed through gentle evaporation under vacuum. Preformed MRPs from all the combinations were mixed thoroughly. The flasks were heated on a boiling water bath for 35 min and cooled. One set of samples along with control was taken immediately for analysis of TBARS, peroxide value and gas chromatographic profile, while the other 3 sets were kept at 5° C to monitor the storage changes in the model system. All the experiments were repeated 5 times and the values were expressed as mean ± SD.

3.3. Sample codes for the methyl linoleate model system studies and antioxidant assay

These were: 30 mM/0h, A; 30 mM/1h, A1; 30 mM/2h, A2; 60 mM/0h, B; 60 mM/1h B1; 60 mM/2h, B2 and control, CT; glucose+glycine, T1; glucose + tryptophan, T2; glucose + methionine, T3; glucose + aspartic acid, T4 and glucose + lysine, T5.
3.4. Meat / poultry samples

Fresh mutton (sheep meat), beef and pork (leg portion, 2-3h post mortem) and chicken legs (2-3 h post mortem) were purchased from the local market, washed thoroughly under running water deboned and cut into small pieces (1.5cm x1cm) and used for processing. For the evaluation of antioxidant potential and WOF, minced meat was employed. For Hurdle processing and irradiation studies chicken legs (drumstick pieces) with bones were employed in the study.

3.5. Sample preparation for Fluidised bed drying

Various green spices, e.g. onion, garlic, ginger, green chillies and turmeric, pepper, cloves, cinnamon, cardamom and cumin, were obtained from the local market.

Wet masala paste was prepared by using onion, garlic, ginger, green chillies and turmeric powder. Acidity and salt were adjusted. Masala was fried in vegetable oil and divided into five equal parts.

One portion of mutton sample was cooked with the above prepared masala (sample A). Another portion was cooked with the preformed MRPs and masala (sample B). Third portion was cooked with the masala and spices (cloves, cinnamon, cardamom and pepper) (sample C), a fourth portion was cooked with masala and ascorbic acid (sample D) while the last and fifth portion was cooked with masala, spices, MRPs and ascorbic acid (Sample E).

3.6. Freezing and Fluidised bed drying

All the five samples were separately frozen in a blast freezer (Hull corp. USA) at −35°C for 3h. The samples after uniform freezing were subjected to drying in a fluidised bed drier (M/S Chemech Industries, Mumbai) at 75°C for 6h. The product after FT/FBD drying were packed in PFP (45 GSM paper/ 20μ Al foil / 37.5 μ LDPE) and stored to assess the shelf stability at ambient temperature (25 ± 2°C).
3.7. Sample preparation for the evaluation of antioxidant potential and WOF of synthetic and natural antioxidants.

Synthetic antioxidants like TBHQ, BHA and PG were incorporated at the permitted level (0.02%). Natural antioxidants like MRP (60 mM/2 h) ascorbic acid (500 ppm) and finely powdered spices (250 mg/100 g) like cinnamon and cloves were separately added into minced meat samples and thoroughly mixed. Like this another three sets of samples were prepared for evaluation after 2, 4 and 6 days storage at 5°C. Treated samples were packed in polypropylene pouches. These were coded as sheep control (SC), beef control (BC), pork control (PC), cloves (T1), cinnamon (T2), MRP (T3), Ascorbic acid (T4), TBHQ (T5), BHA (T6) and PG (T7).

3.7.1. Cooking and storage of samples

All the meat and poultry samples after treatment with natural and synthetic antioxidants (4 sets) were packed in polypropylene pouches (15"x9") and cooked in boiling water bath under atmospheric pressure for 35 min and cooled to room temperature. One set of samples was taken for immediate analysis and the other three were kept at 5°C for evaluation after 2, 4 and 6 days. All the experiments were repeated 5 times on the different set of samples and the values are expressed as mean ± SD.

3.8. Sample preparation for the preparation of F.D chicken with antioxidants

Wet masala paste was prepared by using onion, garlic, ginger, green chillies and turmeric powder. Acidity and salt were adjusted. Masala was fried in vegetable oil and divided into five equal parts.

One portion of chicken sample was cooked with the above-prepared masala (sample 1). Another portion was cooked with the preformed MRP and masala (sample 2). Third portion was cooked with the masala and spices (clove, cinnamon and cardamom) (sample 3). Fourth portion was cooked with masala and ascorbic acid (sample 4) and the last and fifth portion was cooked with masala, spices, MRP and ascorbic acid (sample 5).
3.9. Freeze-drying

Freeze-drying was carried out in a pilot scale freeze dryer (Hull Corporation, USA), capable of rapid freezing. The samples were pre-frozen to 

\(-35 \pm 2^\circ C\) in stainless steel trays. Drying was carried out under chamber pressure in the range of between 100 to 300 microns, using plate temperature of 65\(^\circ\) C. The samples were dried to 5\% moisture. When the product was dried to required moisture level, nitrogen gas was used to break the vacuum. The dehydrated products were removed from the drier and taken to the humidity and temperature controlled (20 \(\pm\) 2\% RH and 25 \(\pm\) 2\(^\circ\) C) room for packing.

3.10. Hurdle processing of chicken legs

Thoroughly cleaned chicken legs were marinated well for a definite period (2.5 – 3h) in lemon juice (pH 2.9 – 3.3) containing chilly and turmeric powders.

3.10.1. Wet masala preparation

Wet masala ingredients after cleaning and decontamination were ground into a paste and the paste was heat-treated for 45 – 55 min with continuous stirring at 95 to 100\(^\circ\) C. Masala was then mixed with requisite quantities of spice powders (clove, cinnamon, cardamom, jeera, coriander and salt) and stirred well for 10 to 15 min. Hydrogenated fat was incorporated at this stage, again and mixed well and heated for 10 to 15 min at 120 to 130\(^\circ\) C.

3.10.2. Cooking, coating of masala with fat and surface dehydration

Marinated chicken legs were divided into three portions and cooked separately. One portion as such, second portion with preformed MRPs (60mM/2h) and the third portion with ascorbyl-6-palmitate (500ppm) for 30-45 min.
The cooked chicken legs were then coated with the spice masala containing fat and heated at 100 to 110°C for 10 to 15 min. After cooking with masala and fat, the pieces was subjected to surface dehydration at 80 to 90°C for 60 to 70 min in a hot air oven.

After surface dehydration, the leg pieces were cooled to ambient temperatures and packed in paper / foil / polyethylene packages (45 GSM paper / 20μ Al. foil / 37.5μ low density LDPE). Packed products were taken for further studies of irradiation and stored at ambient temperature (25 ± 2°C, RH 60 to 70%) and at 5 ± 2°C (RH 80%) for chemical evaluation of the products.

3.11. Irradiation of poultry and meat samples

The samples were subjected to γ-irradiation at melting ice temperature (1 – 3°C) in a package irradiator (Nordion Intl. Inc. Ontario, Canada) with a 60Co source. The samples received minimal doses of 1, 2 and 3 KGy for poultry samples and 1, 2, 3 and 4 KGy for meat samples. Dosimetry was performed by a Cerric-Cerrous dosimeter calibrated against Fricke’s dosimeter. Dosimetry inter comparison was carried out with National standards established by the Radiological Physics and Advisory Division (RP and AD), Bhabha Atomic Research Centre (BARC), Mumbai, India.

3.11.1. Sample code for the studies on the effect of MRPs and ascorbyl 6-palmitate on the lipid oxidative profile of hurdle processed irradiated chicken legs

In this study three dosage levels of irradiation has been employed. 1, 2 and 3 KGy along with non irradiated sample (0 KGy). Each category three samples i.e., a, b and c. a-control, b-treated with MRP and c-treated with ascorbyl 6-palmitate. Thus there are 12 samples for analysis i.e., 0a, 0b, 0c, 1a, 1b, 1c, 2a, 2b, 2c, 3a, 3b, and 3c.
3.12. Analytical methods

3.12.1. Moisture estimation (Oven drying method)

The moisture content of FBD mutton and hurdle processed chicken samples were determined using the standard AOAC (1984) procedures.

5-10 g of samples was taken in a previously heated and tared flat bottom aluminium dishes containing thin layer of asbestos. After weighing the dish cover was removed and kept for drying in an oven at 100°C for 6 - 8h. The dried sample were cooled in a desiccator for 30 - 45 min and weighed. The moisture content of the samples was calculated as follows.

\[
\text{Moisture content} = \frac{100 (W_i - W_2)}{(W_1 - W)}
\]

Where, 
\( W = \) Weight of empty dish
\( W_i = \) Weight of dish before drying.
\( W_2 = \) Weight of dish after drying.

3.12.2. Moisture estimation (Vacuum oven method)

Moisture content of freeze dried chicken samples was determined using vacuum drying to a constant weight as per Ranganna (1995).

2-3g of dried sample was taken in a previously tared flat bottom dish in de-humidified room where relative humidity was maintained at 23 ± 2% to avoid moisture uptake. Dish was covered with a lid and kept it in a desiccators containing P₂O₅ and transferred quickly to a vacuum oven for drying after uncovering the dish. The product was heated under vacuum for 12 to 16h at 70°C. After drying, vacuum was broken through the trap containing calcium oxide. The product was taken from the oven and stored for 30 min in desiccators and weighed. The moisture content of the sample was calculated as above.
3.12.3. Determination of fat content (Soxhlet method)

Fat content of dehydrated meat and poultry samples was determined as per AOAC (1990). The fat was determined by extracting 5-10g of moisture free sample with petroleum ether for 6-8 h in a soxhlet apparatus. The solvent was evaporated by heating at 60°C using flash evaporator and the fat content was determined. The weighing was repeated until a constant weight was obtained. Increase in the weight of tared flask after complete evaporation of petroleum ether indicates the weight of fat.

\[
\text{% of fat} = \frac{\text{Increase in weight of the flask} \times 100}{\text{weight of sample}}
\]

3.12.4. Determination of fat (Folch method)

Hurdle processed meat/poultry samples and other cooked meat samples were subjected for lipid extraction initially and during storage as per the Folch’s procedure (Folch et al., 1957) with a slight modification.

25g of each sample was weighed into a clean and dry beaker. To this 75ml of chloroform: methanol (1:1) v/v was added. It was blended thoroughly in a mixer. It was then filtered through a stainless steel strainer.

The residue ground in a pestle and mortar with a second lot of 75ml Chloroform:Methanol mixture (2:1) v/v and filtered again. The filtrates were pooled together and then filtered through a Whatman No. 41 filter paper. The residue was discarded.

The filtrate was taken in a separating funnel to which 25ml of 0.88% aqueous potassium chloride solution and 12.5ml of methanol was added and shaken well. The solvent layer after separation again shaken with 25ml of solvent mixture (CHCl₃: CH₃OH: 0.74% KCl) (3:48:47) v/v/v. The solvent layer was collected, added with 4 to 5g of anhydrous sodium sulphate, kept for 5 min and filtered through Whatman No 41 filter paper. Solvent was evaporated using a rotary vacuum evaporator. The lipid obtained was weighed and the percentage of lipid obtained was calculated as above.
3.12.5. Total protein in meat samples (AOAC 1984)

One gram of meat sample was weighed and transferred to a 250ml Kjeldahl flask taking care to see that no portion of the sample clinged to the neck of the flask. Approximately 1g of catalyst mixture (2.5g SeO₂, 100g K₂SO₄ and 20g CuSO₄ · 5H₂O) and 25ml of concentrated H₂SO₄ were added and placed the flask on the stand in the digestion chamber. The flask was heated gently over a flame until the initial frothing ceased and the mixture boiled briskly at a moderate rate. The digestion continued until the digest becomes clear. The digest was cooled and made up to 100ml in a volumetric flask with distilled water. A blank digestion was also carried out without the sample and made up the digest to 100ml.

1ml aliquot was distilled with the addition of 2ml of 30% NaOH solution. The ammonia liberated was absorbed into 5ml of a 2% solution of boric acid containing a mixed indicator of bromocresol green and methyl red. The content s of the flask were titrated against 0.01N HCl to bluish green to pink colour. The blank distillation and titration were carried out as in the case of the sample.

\[
\text{Nitrogen} \% = \frac{\text{(Sample titre - blank titre) x N of HCl x volume made up of the digest x 100}}{\text{Aliquot of the digest taken x wt of the sample taken x 1000}}
\]

\[
\text{Protein} \% = \text{Nitrogen} \% \times 6.25.
\]

3.12.6. Total ash

The tare weight of silica dishes (7 – 8 cm dia) was noted. Five g of the sample was weighed out into each dish and ignited the contents on a Bunsen burner. The material was ashed not more than 525°C for 6h in a muffle furnace, cooled the dishes and weighed. The difference in weight gave the total ash content and was expressed as percentage (Ranganna, 1995).
3.12.7. pH measurement

The pH of T1, T2, T3, T4 and T5 under the different concentration time combinations of MRPs – A, A1, A2, B, B1 and B2 of methyl linoleate model studies and samples were measured initially and after heating with the help of a Eutech instrument (Cyberscan pH 510 pH/mv/temp) which was calibrated earlier with a pH 4.0 buffer solution.

3.12.8. Optical density measurement

Optical density measurements for all the concentration of MRPs (A, A1, A2, B, B1, and B2) under different treatments (T1, T2, T3, T4 and T5) were carried out using a Chemito UV visible spectrophotometer model 160, Chemito instruments, India. Non-enzymatic browning index was measured at 420 nm.

3.12.9. Peroxide value by colorimetric method

Peroxide value of methyl linoleate model study samples was estimated colorimetrically by the method of Osawa and Namiki, (1981).

The test sample was treated with 5ml of 99.5% ethanol, 8ml of 0.05M-phosphate buffer (pH 7.0) and 4ml of distilled water and was placed in a vial with a screw cap and placed in an oven at 40° C in the dark. To 0.1ml of this sample solution, were added 9.7ml of 75% ethanol and 0.1ml of 30% ammonium thiocyanate. Precisely 3min after the addition of 0.1ml of 2X10⁻² M ferrous chloride in 3.5% hydrochloric acid to the reaction mixture, the absorbance of the red colour developed was measured at 500 nm. From the absorbance, the peroxide value was calculated and expressed as mg/kg of the sample.

3.12.10. Peroxide value by titrimetric method

Peroxide values of the samples were determined as per AOAC (1984). 0.5 to 1g of extracted fat was dissolved in 30ml glacial acetic acid and 20ml Chloroform into a clean 250ml Iodine flask. 0.5ml of saturated potassium iodide was added to the flask and kept in dark after nitrogen flushing for 15 min. 50ml distilled water was added and titrated against 0.02N sodium
thiosulphate solution using starch as an indicator. From the titre value, PV of
the sample was calculated as follows and expressed as milli equivalent of
peroxide per kg of fat.

\[
PV = \frac{V \times N \text{ of } \text{Na}_2\text{S}_2\text{O}_3 \times 1000}{\text{Weight of sample}}
\]

3.12.11. Free fatty acid content (FFA)

The FFA content of the samples was estimated as per AOAC (1990). A
known quantity of fat extracted was taken in to a 100 ml flask and 50 ml of
hot neutralised alcohol was added followed by 1 -2 ml of phenolphthalein
reagent. The flask was shaken vigorously to dissolve all the fat content and
titrated against 0.25 N NaOH solutions to get a faint pink colour. From the
titre value FFA content was calculated as follows.

\[
\text{Free fatty acid (\% as oleic acid)} = \frac{\text{ml of alkali} \times N \text{ of alkali} \times 28.2}{\text{Weight of fat}}
\]

3.12.12. Thiobarbituric acid Reactive substance (TBARS) determination

TBARS values in meat/poultry samples were determined as per
Taraldgis method (1960).

0.02 M 2-Thiobarbituric acid in 90 % glacial acetic acid was prepared
by warming on a boiling water bath.

**TEP standard preparation:** 1 x 10^-3 M solution of 1, 1, 3, 3
Tetraethoxy propane was prepared in distilled water. Appropriate
concentration of TEP was prepared from the main stock solution. Colour
development was carried out using TBA reagent at various concentrations of
the standard to draw the standard TEP curve as shown in Fig-3.
3.12.12.1. Sample distillation and estimation

Taraldgis method (1960) is one of the most widely used tests to evaluate the extent of lipid oxidation in meats. This is based on the reaction between important oxidation product malonaldehyde with TBA reagent to produce a colour complex. The chromogen results from the condensation of 2 molecules of TBA with 1 molecule of malonaldehyde. This reaction is indicated in Fig-4.

20 g of blended sample was accurately weighed and transferred into a RB flask. To that 2.5 ml of con.Hcl was added along with 97.5 ml of distilled water. pH was adjusted to 1.5. Mixture was steam distilled and 50ml distillate was collected in 10 min. From this 25 ml of distillate was transferred into stoppered glass tubes and 5ml of TBA reagent was added. The test tubes were kept in boiling water bath for 35 min and it was cooled and OD was measured at 538nm. The TBARS values were calculated using the standard curve.
3.12.13. Warmed over flavour/ Total carbonyls

WOF profile expressed in terms of mg of n-hexanal / 100g fat was monitored by the method of Benca and Mitchela (1954).

Powdered/ground sample about 5g were extracted in 50 ml of carbonyl free benzene (to one litre of benzene, 5g of Dinitrophenyl hydrazine (DNPH) and one g of trichloroacetic acid were added. The contents refluxed for an hour and distilled) by shaking in mechanical shaker for an hour. Into 50 ml volumetric flask, 5 ml sample filtrate and 3 ml of 3 to 4% trichloracetic acid (in benzene) and 5 ml of 0.05% DNPH solution (in benzene) were added and incubated at 60° C for half an hour to convert free carbonyl into hydrazones. After cooling for 10 min, 4 % alcoholic potassium hydroxide were added and volume was made up to 50 ml with carbonyl free ethanol (to 1 litre of alcohol. 7g of aluminium powder and 10g of potassium hydroxide were added. The contents were refluxed and distilled). After 10 min, absorbance was read at 480 nm. Blank were prepared in the same manner substituting 5ml benzene instead of sample extract.
A standard curve was drawn using hexanal (50 to 250 mg) in 5 ml benzene instead of sample extract. Total carbonyls were calculated with the help of standard curve and expressed as mg of hexanal/100g of fat.


Ferrous iron reacts over a wide range of pH with 1, 10 – phenanthroline to form a deep reddish range colour complex. Total iron is obtained in the sample by using acid to dissolve precipitated iron and by applying hydroxylamine hydrochloride to reduce ferric iron to the ferrous state.

A stock solution was prepared by dissolving 0.7022 g of ferrous ammonium sulphate in about 700 ml of distilled water containing 10ml of concentrated reagent grade sulphuric acid. The solution is diluted to one litre in a volumetric flask. 1ml of this stock solution contains 0.1 mg of iron. For drawing the standard graph, appropriate concentrations was prepared from the stock solution and it was made to react with 1,10 – phenanthroline reagent in the presence of acetate buffer and the colour readings were measured at 510 nm. The concentrations of the aliquots taken for the standard solution were plotted against the corresponding OD and a standard graph is plotted.

One g of meat sample was weighed and kept for ashing in porcelain dish in muffle furnace. It was cooled and little water was added, 1 ml of con. HCl and 1 ml of hydroxylamine hydrochloride were added. The ash was dissolved and transferred to the flask. It was washed with hot water and made up to 15 to 25 ml. Then transferred to Nessler tubes. 10 ml of acetate buffer and 1ml of 1, 10 – phenanthroline solution were added and mixed well. The solution was allowed to stand for 15 min for the development of colour. A blank was run with water, acetate buffer and 1, 10 – phenanthroline solution. Then OD was taken at 510 nm. It was calculated from the standard curve and reported as total iron in mg/100g (Edward Koniccko, 1979).

3.12.15. Non-heme iron estimation

The catalytic activity of non-heme iron in the presence of antioxidants was estimated by the method of Igene et al (1985). The estimation was...
carried out by first precipitating the bound heme iron with 10 ml of 40% trichloro acetic acid, following centrifugation. The supernatant was removed for determination of free non-heme iron by the colorimetric method using 1,10-phenanthroline as above and calculated the non-heme content from the standard graph.

3.12.16. Antioxidant assays
3.12.16.1. Antioxidant activity by TBARS method

The antioxidant potential expressed in terms of percentage of antioxidant activity was calculated by the equation Wijewickreme and Kitts, (1998).

\[
\% \text{ AOA} = \frac{[\text{TBARS value of the control} - \text{TBARS of the test sample}] \times 100}{[\text{TBARS value of the control}]}
\]

TBARS values were expressed as mg malonaldehyde/kg sample was estimated colorimetrically using 2-thio barbituric acid (Taraldgis et al., 1960) as described earlier.

This method was employed to evaluate the antioxidant activity of natural and synthetic antioxidants in different species of meat.

3.12.16.2. Antioxidant assay for DPPH radical – scavenging activity

The scavenging effects of samples of DPPH radical were monitored according to the method of Yen and Chen (1995). Briefly a 2.0 ml aliquot of test sample (in methanol) was added to 2 ml of 0.16 mM DPPH methanolic solution. The mixture was vortexed for 1 min and then left to stand at room temp for 30 min in the dark, and its absorbance was read at 517 nm. The ability to scavenge the DPPH radical was calculated using the following equation.

\[
\text{Scavenging effect (\%)} = \left( \frac{1-(A \text{ sample} - A \text{ sample blank})}{A \text{ control}} \right) \times 100
\]
Where the A control is the absorbance of the control (DPPH solution without sample), the A sample is the absorbance of the test sample (DPPH solution plus test sample) and the A sample blank is the absorbance of the sample only (sample without DPPH solution).

To evaluate the antioxidant activity of different concentrations and heating time of MRPs, DPPH radical scavenging activity method was employed.

3.12.16.3. Antioxidant assay with β-carotene-linoleic acid system

The antioxidant activities of samples assayed using the β-carotene-linoleic acid system were measured as in the method of Jayaprakasha et al., (2001). Briefly 4 ml of a solution of β-carotene in chloroform (1 mg / ml) were pipetted into a flask containing 40 mg of linoleic acid and 400 mg of Tween-40. The chloroform was removed by rotary evaporator under vacuum at 45° C for 4 min and 100 ml distilled water were added slowly to the semisolid residue with vigorous agitation to form an emulsion. 50 μl of test sample and 200 μl of the emulsion is mixed and the absorbance was measured at 450 nm, immediately against a blank consisting of the emulsion without β-carotene. The sample was kept at room temperature (28 ± 3° C) and the absorbance measurements were conducted again at 30 min intervals up to 420 min. All determinations were carried out in triplicate. The antioxidant activity of MRPs were evaluated in terms of bleaching of β-carotene using the following formula:

$$A_{\text{Antioxidant Assay}} = \left[ 1 - \frac{(A_0 - A_1)}{(A'_0 - A'_1)} \right] \times 100$$

Where $A_0$ and $A'_0$ are the absorbance of values measured at zero time of the sample and the control, respectively, and $A_1$ and $A'_1$ are the
absorbances measured in the test sample and the control respectively, after 420 min.

3.12.17. Determination of reducing power

The reducing power of all samples of MRPs was determined as described by Dorman et al., (2003). Generally 1 ml of each sample dissolved in distilled water was mixed with 1 ml of phosphate buffer (0.2M, pH 6.6) and 2.5 ml of a 1% aqueous potassium hexacyanoferrate [K₃Fe(CN)₆] solution. After a 30 min incubation at 50°C, 1.5 ml of 10% trichloroacetic acid was added and the mixture was centrifuged for 10 min. Finally 2 ml of the upper layer was mixed with 2 ml of distilled water and 0.5 ml of 0.1% aqueous FeCl₃ and the absorbance was recorded at 700 nm. The data were presented as ascorbic acid equivalent (ASCAE) in mg of ascorbic acid per g of sample. A greater value of the ASCAE related to greater reducing power of the sample.

3.12.18. Critical water content and water activity of the product

Sorption characteristics of the dehydrated FT/FBD samples and FD chicken samples were determined by the method of Resnik et al., (1984). 2-5 g of sample were placed in a 3” dia petri dish and exposed to different relative humidity conditions, ranging from 0 to 85%, using various salt solutions in desiccators. Changes in weight of the samples were recorded at hourly interval in the first 24 h and later, at one day intervals, up to seven days, followed by weekly intervals until equilibrium was achieved. These data were used to evaluate the equilibrium moisture content and water activity of the product (Brunauer et al., 1938).

3.13. Sensory evaluation

The sensory characteristics of the products with different combinations were evaluated initially and during storage for its quality attributes like colour, aroma, texture, taste and overall acceptability on a 9 point hedonic scale by a panel of judges, keeping 9 for excellent and 1 for very poor as per the method of Murray et al., (2001).

Differential scanning calorimetry was used to determine the onset of endothermal change in specific heat (Tg) and isothermal crystallization of meat products containing various additives, using the Differential scanning calorimeter, Model 2010 fitted with graphic plotter and the Thermal Analyser (TA instruments, USA) as per the procedure described by Jagannath et al., (1999b) for Tg and Krause et al., (1984) for isothermal crystallization experiments. 5 -10 mg samples were weighed into standard aluminium pans in a dehumidified room to avoid moisture uptake along with empty pan. The hermetically sealed sample pan was held at a temperature, low enough to ensure no crystallisation for 1 min. The sample was then ramped up to a maximum temperature of 320° C. The final isothermal holding temperature where it was held for 20 min during which time crystallisation was observed and crystallisation curves were recorded with respect to time. Taking the end of the induction period as zero time, the peaks were integrated with respect to time. Degree of crystallisation was plotted against time for each peak. The results were analysed using the Universal Analysis 2000 software for windows 95/98/NT, version 3.0 TA Instruments Inc. First the onset temperature and completion temperature were determined the peak is integrated with respect to temperature between the start and stop base line limits. The programme uses the sample size to normalise the area under the peak and obtained the experimental heat (ΔH) in J/g. Taking the end of the induction period as zero time, the peaks were integrated with respect to time. Degree of crystallisation was plotted against time for each peak. The results were analysed using the Avrami equations (Avrami 1939) as follows.

\[ Q(t) = 1 - \exp(-KF^n) \]

Where \( Q(t) \) is the relative crystallisation at time \( t \), and \( k \) is the isothermal crystallisation rate, \( n \) is the Avrami index using the method of least squares \( K \) and \( n \) values were obtained.
3.15. UV-Visible spectrum of MRPs

UV-visible spectrum of the 60 mM/2h concentration of the MRPs was recorded using spectronic double beam UV-visible spectrometer model "GENESIS 2". Spectrum was recorded at medium speed with a resolution of 1nm from 200 to 500nm. Quartz cuvettes were used for holding the samples. Blank is subtracted from the sample spectrum automatically.

3.16. Total volatile content

Total volatile content of the samples was estimated as per Weurman (1969). 100g of dehydrated samples were taken in a simultaneous steam distillation evaporation flask and connected to a distillation head. 100ml of dichloromethane was taken in a 500 ml round bottom flask and connected to receiving end of the distillation flask. The distillation was carried out for two h and the solvent containing flavour compounds was dried over anhydrous sodium sulphate, evaporated and weighed. The total volatile content was calculated as follows.

\[
\text{Total volatile content mg/kg} = \frac{1000 \times \text{weight of the volatile}}{\text{Weight of sample}}
\]

3.17. Gas liquid chromatography

3.17.1. Gas chromatographic determination of methyl linoleate degradation in the presence of MRPs.

Methyl linoleate degradation was determined by gas chromatographic analysis using a Chemito, Model 8610 HT Gas chromatograph fitted with a 10 X 1/16 " SS column packed with 'Famex' with a flame ionisation detector and a computerised integrator Chemito 5000 data processor. The analysis was carried out isothermally at 190° C. Injector and detector port temperatures were maintained at 220° C and 230° C respectively. The flow rate of carrier gas (nitrogen) was maintained at 45 ml/min and that of hydrogen at 40 ml/min. The percentage loss of methyl linoleate was calculated by comparing the area of the standard methyl linoleate peak.
3.17.2. Total fatty acid profile of meat/poultry samples by Gas chromatographic method

3.17.2.1. Esterification of fatty acids

The samples were esterified as per the procedure of Metcalfe et al., (1966) with slight modifications.

About 150 mg of lipid was accurately weighed into a clean and dry stoppered test tube. 4 ml of 0.5 N alcoholic sodium hydroxide solutions was added and heated for 5 min over a water bath at 90°C. On cooling 5 ml of Boron trifluoride-methanol reagent (14%) was added and heated for 5 min at 90°C over a water bath, followed by addition of 10 ml of saturated sodium chloride solution. The samples were thoroughly cooled to room temperature and 5 ml of hexane was added to each tube. It was shaken well and kept undisturbed. The upper hexane layer was drawn out into clean dry conical flask and dried over anhydrous sodium sulphate to remove the traces of moisture if present. The samples were filtered and transferred to stoppered clean dry tubes for gas chromatographic analysis.

3.17.2.2. Total fatty acid analysis by Gas chromatography

Analysis of total fatty acids was carried out by ceres-600 plus, Chemito model Gas chromatograph fitted with BPX 70 column (25 mt, 0.32 mm ID) and flame ionisation detector. Temperature gradient programming was employed from 150 to 220°C. Split ratio was adjusted to 1:25 and capillary flow of carrier 2 ml/min. Injector and detector port temperatures were adjusted as 230 and 240 respectively. For FID Hydrogen and oxygen were used and the flow was adjusted as 45 ml/min and 450 ml/min respectively. Along with samples standard esters of fatty acids were also injected and the fatty acids were detected by comparing the retention time of the standard esters of fatty acids. The quantification of the fatty acids were carried out by evaluating with the standard fatty acid esters area corresponding to each peak in the chromatogram. Iris-32 software is used to integrate and evaluate the chromatogram in the analysis.
3.18. Statistical analysis

Data obtained were subjected to analysis of variance (ANOVA) and Duncan’s multiple range tests to evaluate the statistical significance of the treatments and significance was established at $p<0.01$. Regression analysis for the correlation between antioxidant activity, warmed over flavour, non-heme iron and total carbonyls were performed and best-fit equations as well as correlation coefficients were established using the software Curve Expert 1.3 (Hyams, 2003).

*All the chemicals and standards used in the above investigations are of highest purity from reputed firms.*