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Abstract

Fats and oils are recognized as essential nutrients in human diets. Nutritionally, they are concentrated source of energy (9 cal/ gram); provide essential fatty acids which are the building blocks for the hormones needed to regulate bodily systems; and are a carrier for the oil soluble vitamins A, D,E and K. They also enhance the foods we eat by providing texture and mouth feel, imparting flavor, and contributing to the feeling of satiety after eating. Oils exposed to high temperature generate hazardous effects. The purpose of this study is to determine the antioxidant stability and chemical characterization of heated and unheated corn oil. The chemical characterization of oil was carried out using GLC-MS analysis. The anti-oxidative properties of heated and unheated corn oil are examined by using different In-vitro antioxidant assays such as 2, 2'-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid (ABTS) and 1, 1'-diphenyl-picryl-hydrazyl free radical (DPPH) scavenging assay. The GLC-MS analysis of oil shows some changes in the content of antioxidants like absence of sitosterol, decanoic acid (unsaturated fatty acid) etc., when exposed to smoke point temperature. The IC_{50} value of in-vitro antioxidant activity also reveals that antioxidant activity was found to be lost when the oil is exposed to higher temperature. In conclusion heating oil to higher temperature was observed to inhibit the potent activity and loss of large number of antioxidants.

Key words: *ABTS, DPPH, Corn oil, GLCMS, sitosterol, stigma sitosterol.*

Introduction

Vegetable oils undergo various reactions like hydrolysis, oxidation, polymerization, isomerisation and various reactions during deep fat frying. Frying conditions however, saturate the unsaturated fatty acids, although the ratio of saturated to unsaturated fatty acids will change due to degradation and polymerization of the unsaturated fatty acids. The frying operation also results in an increase in the level of "polar compounds" (mono- and diglycerides, free fatty acids, and other polar transformation products) formed during frying/heating of foodstuffs in the oil.

It is the usual practice to discard frying fat when prolonged frying causes excessive foaming of the hot oil, the fat tends to smoke excessively, usually from prolonged frying with low fat turnover, or an undesirable flavour or dark colour develops. Any or all of these qualities associated with the fat can decrease the quality of the fried food (1).

Earlier reference mentioned that the more unsaturated the fat or oil, the greater will be its susceptibility to oxidative rancidity.

Predominantly unsaturated oils (i.e., soybean, cottonseed, or corn) are less stable than predominantly saturated oils (i.e., coconut oil). In the present study, corn oil is selected to evaluate the stability by exposing to higher temperature.

Corn oil, cooking oil, is extracted from the germ of corn, has high smoke point (236^0C). Corn oil has a milder taste and is less expensive than most other types of vegetable oils. Refined corn oil is 99 % triglyceride, with proportions of approximately 59 % polyunsaturated fatty acid, 24 % monounsaturated fatty acid, and 13 % saturated fatty acid. It is sometimes used as a carrier for drug molecules in pharmaceutical preparations (2).

The oxidative stability of corn oil is evaluated by carrying out the chemical characterization in GLC-MS analysis and comparing the potent effect of exposing to higher temperature by carrying out various methods of *in-vitro* antioxidant activity.

EXPERIMENTAL

Chemicals and reagents

2, 2'-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid (ABTS) and 1, 1'-diphenyl-2-picryl-hydrazyl (DPPH) free radical were purchased from Sigma chemical company, MO, USA. Other chemicals used were of analytical grade.

Collection of oil: Corn oil has been collected from a local grocery shop located in Thanjavur district of Tamil Nadu, India.

Frying of oil:

Fifty milliliter of the sample oil has been placed in a copper beaker and heated on an electric device, stirring manually with glass rod. A microcontroller based temperature controller has been designed and was used to monitor the sample temperature. To mimic the oil oxidation process during frying, the sample was heated up to 250°C for five times. Initially, the sample was heated to 250°C for half an hour. Then, it was allowed to cool until room temperature is achieved. Similarly, the sample was subjected to heating up to 250°C for 1 hour, 1 ½ hour, 2 hour and 2 ½ hour respectively ensuring that every time the sample is allowed to cool up to room temperature before heating it next time. In order to ensure that the sample was heated to the temperature greater than its smoke point, it has been exposed to successive heating.

$ABTS^{+\bullet}$ radical decolorisation assay $ABTS^{+\bullet}$ radical cation decolourisation is carried out by following the method of Re (3). $ABTS^{+\bullet}$is generated by mixing 2.5 ml of 7 mM ABTS with 14.7 mM ammonium per sulphate and stored in the dark at room temperature for 16 hours. The solution is diluted with water to achieve an absorbance of 0.7 O.D at 734 nm.

$$ABTS + H_2O_2 \longrightarrow ABTS^{+\cdot} + H_20 \text{ ------ (1)}$$

The radical scavenging activity is assessed by mixing 2 ml of this $ABTS^{+\cdot}$ solution with different concentrations of sample dissolved in chloroform (25, 50, 75, 100 µl). 1.0 ml of chloroform along with 2.0 ml of $ABTS^{+\cdot}$ is used as control. The final absorbance is measured at 734 nm. The

antioxidant capacity is expressed as percentage inhibition, calculated using the following formula,

$$\text{Inhibition (\%)} = 100 \times (A_{(cont)} - A_{(Test)})/A_{(Cont)} \text{ ---------- (2)}$$

Where $A_{(cont)}$ is the absorbance of the control, and $A_{(Test)}$ is the absorbance of the sample at 734 nm. IC_{50} is the antioxidant concentration that inhibits the $ABTS^{+\cdot}$ reaction by 50% under the experimental conditions. This is calculated by Graph pad software version 5.0.

$DPPH^{*}$ radical scavenging assay

DPPH radical decolourisation is carried out by following the method of Brand (4). Chloroform solutions of oil at different concentrations (25, 50, 75,100 µg/ml of chloroform) are added to 2 ml of a methanol solution of $DPPH^{\bullet}$ free radical or methanol alone (control). The reaction mixture is shaken by cyclomixer and then kept in the dark for 30 min under ambient conditions.

$$DPPH^{+} + AH \longrightarrow DPPH + A^{+} \text{ --------- (3)}$$

The absorbance is measured at 517 nm, and the capability of scavenge the $DPPH^{+}$ radical is expressed as percentage inhibition, calculated using the following formula,

$$\text{Inhibition (\%)} = 100 \text{ X } (A_{(cont)} - A_{(Test)})/A_{(Cont)}) \text{ ---------- (4)}$$

Where, $A_{(cont)}$ is the absorbance of the control and $A_{(Test)}$ the absorbance of the sample at 517 nm. IC_{50} is the antioxidant concentration that inhibits the $DPPH^{*}$ reaction by 50% under the experimental conditions. The IC_{50} value was calculated using non-linear regression in Graph Pad software version 5.0. Low IC_{50} values indicate high radical scavenging activity of cation. All analyses were run in triplicate and averaged.

Statistical analysis:

All data on total antioxidant activity are the average of triplicate. To examine the effect of type of compound and concentration on antioxidant activity, graph pad software version 5.0 was used (r^2 =0. 0.9949, $p<0.005$, $n>7$). The datas were recorded and analysed by SPSS (version 12).One-way analysis of variance was performed by ANOVA procedures. Table 3 shows the

significant differences between means determined by Duncan's multiple range tests, p-Values <0.05 were regarded as significant and p-value<0.001 were very significant.

GLC-MS analysis

For the fatty acid compositional variation determination, an Agilent gas chromatograph from Hewlett-Packard (Palo Alto, CA, USA) equipped with a HP 5971 MS detector was used. Separations were carried out on an Agilent – Hewlet Packard fused silica capillary column HP-5 (30 m x 0.25 mm I.D.; 0.25 µm film thickness) (Folsom, CA, USA). The GLC-MS interface temperature was maintained at 250ºC. 1µl of both heated and unheated corn oil samples were injected manually in split less mode with injector port temperature at 220 ºC. The helium carrier gas flow rate was 1 ml /min. The column temperature program was as follows: 90 ºC, held for 1 min, 12 ºC min-1 to 150 ºC, held for 1 min, 2 ºC min^{-1} to 230 ºC, held for 3 min, 10 ºC min^{-1} to 250 ºC, held for 25 min**.** The selective ion mode was used in the analysis. Retention time and abundance of the confirmation ions relative to that of quantification ion were used as identification criteria. Mass Charge range was between 50- 500 amu. Oven temperature programme is maintained to the range of temperature 50° -250° C.

Results and Discussion

. The repeated heating of oils in open-air condition generally leads to reactive oxygen species with oxidation effects. Oxidation of fats degrades the organoleptic quality of food, reduces its nutritional value, and products of the oxidation process can participate in the ageing of an organism and in the aetiology of cardiovascular diseases and cancer.

Food manufacturers continue to add antioxidants to minimize lipid oxidation during food poisoning. An antioxidant may be defined as "a substance when present at lower concentrations compared with those of an oxidizable substrate such as fats, proteins, carbohydrates or DNA, significantly delays or prevent oxidation of substrate (5, 6). In general, antioxidant systems prevent reactive species from being formed, or remove them before they can damage vital components of the cell (7).

Antioxidants are classified into two broad divisions, depending on whether they are soluble in water (hydrophilic) or in lipids (hydrophobic).

In general, water-soluble antioxidants react with oxidants in the cell cytoplasm and the blood plasma, while lipid-soluble antioxidants protect cell membranes from lipid peroxidation (8). These compounds may be synthesized in the body or obtained from the diet (9). Oils and fats are an important part of the human diet. Rancidity of lipids in foods produces undesirable effects including loss of fat-soluble vitamins, generation of off-flavours, palatability problems and even production of toxins and cause food poisoning.

***In- vitro* Analysis**

Corn oil is a high quality excellent frying and resistance to smoke or discolouration oil. Single electron transfer reactions ($ABTS^{\bullet}$ and $DPPH^{\bullet}$) can be relatively slow and measure relative percent decrease in product rather than kinetics or total antioxidant capacity (10, 11). Various phyto-constituents of both the oil probably provide electron to $ABTS^{\bullet}$ and $DPPH^{\bullet}$ thereby causes the decolorisation. The DPPH and ABTS systems have both been commonly used to measure the total antioxidative status of various biological specimens because of their good reproductibility and many quality controls (4).

Both methods apply decolourisation assays to monitor the decrease in their absorbance at characteristic a wavelength during the reaction. For example, the DPPH and ABTS radicals respectively absorb at 517 and 734 nm. When an antioxidant is added to the radicals, there is a degree of decolourisation owing to the presence of antioxidant which reserves the formation of the ABTS radical cation and DPPH radical. It is well known that ABTS activity is closely correlated to DPPH ($r=0.949$, $P<0.001$, $n=9$) because both are responsible for the same chemical property of hydrogen or electron donation (12)

Figure 1 illustrates the scavenging activity of cation is shown by increasing the concentration of oil decreases the colour of both $ABTS^{\bullet}$. Table 1 shows the unheated corn oil can inhibit $ABTS^{\bullet}$ radical cation at the lowest concentration (25 µg/ml) effectively when compared to the heated corn oil. The free radical scavenging activity of both unheated and heated corn oil increases along with the concentration. At the highest concentration (100µg/ml) the activity of both corn oils are at 92 %.

The IC_{50} of unheated and heated corn oil in $ABTS^{\bullet}$ radical decolorisation is calculated as 25.69 µg/ml and 39.55 µg /ml respectively.

Similar figure 2 shows the scavenging activity of heated and unheated corn oil using DPPH method. At concentration from 25 µgm /ml to 100 µgm /ml, the gradient of the curves of percentage of inhibition versus concentration for unheated is steeper than for heated, indicating that in this concentration the anti-radical activity increased rapidly with concentration. Table 1 shows the results that have been observed for effect of corn oil in $DPPH^{\bullet}$ radical decolourisation. The DPPH scavenging activity of heated and unheated corn oil also increases with concentration. The IC_{50} of unheated and heated corn oil in $DPPH^{\bullet}$ decolourisation is calculated as 28.41 µg/ml and 49.34 µg/ ml respectively.

The potent antioxidant activity of unheated corn oil might be due to the presence of antioxidant like sitosterol and stigma sterol.

The synergistic antioxidant activity of sitosterol along with Vitamin E and squalene has been reported earlier by Finotti (13). Marta (14) has reported that the sitosterol thwart the impairment of the glutathione/oxidized glutathione ratio induced by phorbol esters in RAW 264.7 macrophage cultures.

The result of the present study is also supported with the GLC-MS analysis by the reduction % in sitosterol and stigma sterol.

GLC-MS analysis

GLC-MS analysis showed the presence of Decanoic acid, hexa decanoic acid, and sitosterol, octadecanoic acid in unheated oil and hexadecanoic acid alone in heated oil. This result showed that oil when exposed to high temperature or fat frying might result in the polymerization or degradation of various important antioxidants like sitosterol (Fig 3, 4, 5, 6, 7, 8and 9).

This result showed that oil when exposed to high temperature or fat frying might result in the polymerization or degradation of various important antioxidants like sitosterol. Since the antioxidant activity of sitosterol has been reported earlier, an attempt was made to evaluate the antioxidant effect of temperature exposure in corn oil.

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Table 1.ABTS and DPPH radical scavenging effect of antioxidants in heated and unheated of corn oil:

Conc of oil µg/ml)	**ABTS**		**DPPH**	
	heated	**unheated**	**heated**	**unheated**
25	30.8 ± 2.6	44.9 ± 0.7	14.2 ± 3.1	41.7 ± 0.9
50	61.5 ± 1.1	69.9 ± 0.7	52.3 ± 1.3	62.8 ± 0.9
75	73.5 ± 1.2	80.8 ± 1.1	67.2 ± 1.4	76.1 ± 1.3
100	92.7 ± 0.2	88.7 ± 0.6	90.9 ± 0.3	85.9 ± 0.8
IC_{50}	**39.35**	**25.69**	**49.34**	**28.41**

Table 2 Composition of Corn oil before and after heating:

S.No.	**Peak Name**	**unheated oil**		**heated oil**	
		RT	**%Peak area**	**RT**	**%PeakArea**
1	1,4-Undecadiene, (E)- Formula: $C_{11}H_{20}$ MW: 152	2.78	13.85	2.78	22.92
2	n-Hexadecanoic acid Formula: $C_{16}H_{32}O_2$ MW: 256	4.23	10.73	5.85	21.82
3	9,12-Octadecadienoic acid (Z,Z)- Formula: $C_{18}H_{32}O_2$ MW: 280	6.62	43.27	6.6	40.36
4	á-Sitosterol Formula: $C_{29}H_{50}O$	5.85	14.77	10.32	7.66

	MW: 414				
5	Stigma sitosterol-7,22-dien-3-one Formula: $C_{29}H_{46}O$ MW: 410	12.9	17.39	12.25	7.24

Table3: Comparison of ABTS and DPPH method between different groups using one way ANOVA (Group I: Corn oil unheated (ABTS); Group II: corn oil heated (ABTS); Group III: corn oil unheated (DPPH); Group IV: corn oil heated (DPPH).

Con (µg/ml)	Corn oil unheated (ABTS)	Corn oil heated(ABTS)	Corn oil unheated (DPPH)	Corn oil heated (DPPH)	**P value**	F/df
25	44.10±0.522***	30.85±0.745###	41.68±0.647^{aaa}	14.23±0.924fff	0.000	3505.05/9
50	69.97±0.447***	61.549±0.819##	62.75±0.555^{aaa}	52.31±1.016fff	0.000	2910.82/9
75	80.775±0.900***	73.546±0.149###	76.16±0.915^{aaa}	67.19±0.185fff	0.000	1507.19/9
100	88.674±0.745***	92.697±0.759###	85.95±0.852^{aaa}	90.94±0.942ff	0.000	1028.25/9

The values are mean± SD; Statistical analysis was done by one-way ANOVA and post-hoc by Duncan multiple comparison tests. The * mark indicates comparison with group I and group II the # mark indicates comparison with group III and group IV. * P < 0.05; ** P <0.01; *** P <0.001; # P < 0.05; ## P <0.01; ### P <0.001. The f indicates comparison with group I and III and the **a** indicates comparison with group II and IV. f P < 0.05; ff P <0.01; fff P <0.001; **a** P < 0.05; **aa** P <0.01. Basal means just prior to standing; (n=25 in group III at 31st week).

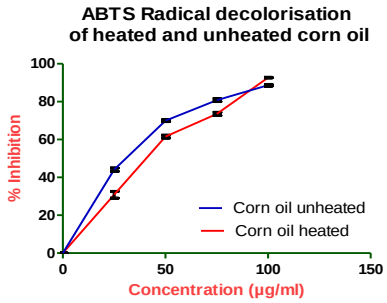

Fig1ABTS radical decolorisation of heated and unheated corn oil

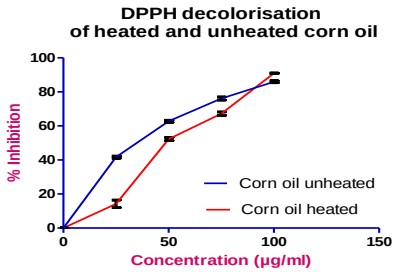

Fig 2DPPH radical decolorisation of heated and unheated corn oil

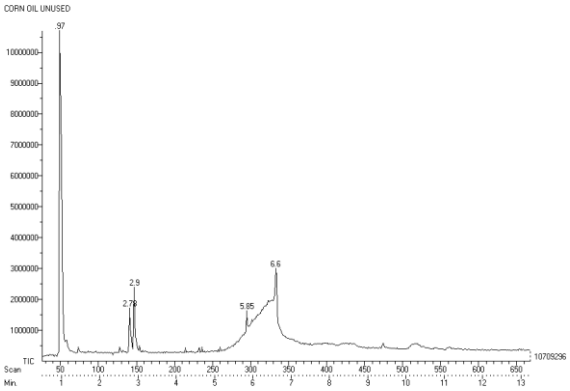

Fig 3 GLC Chromatogram of unheated corn oil

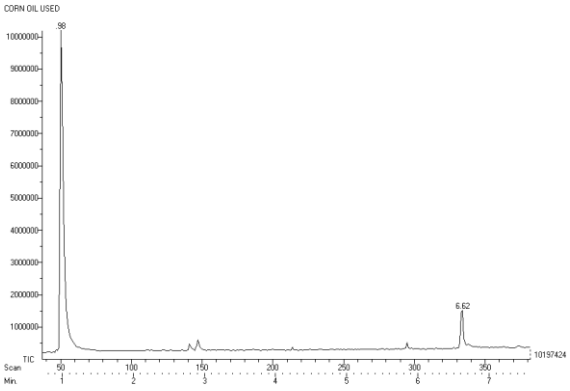

Fig 4 GLC Chromatogram of used corn oil

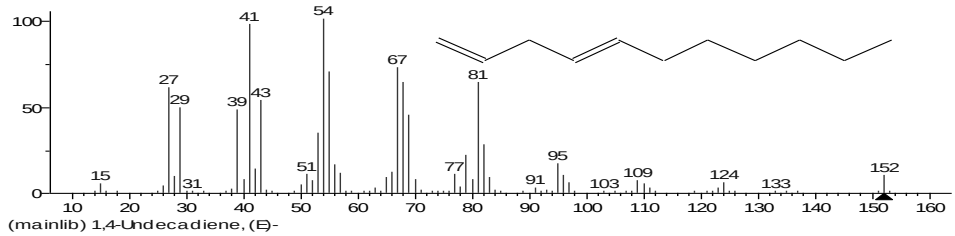

Fig 5 Mass spectrum of 1, 4 undecadiene

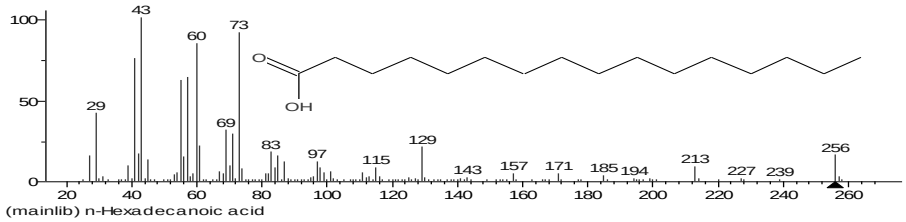

Fig 6 Mass spectrum of hexadecanoic acid

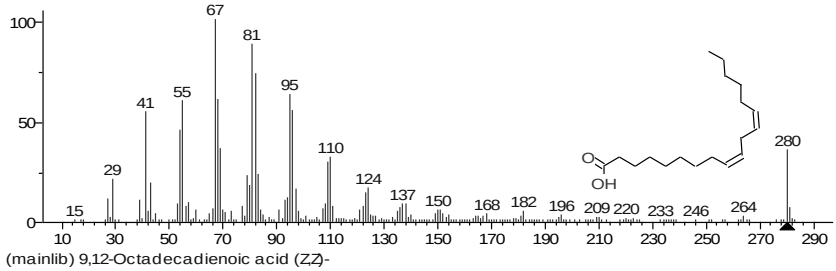

Fig 7 Mass spectrum of 9, 12 – Octadecadienoic acid

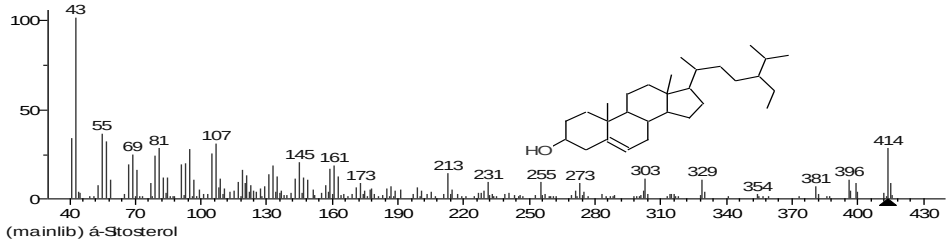

Fig 8 Mass spectrum of Sitosterol

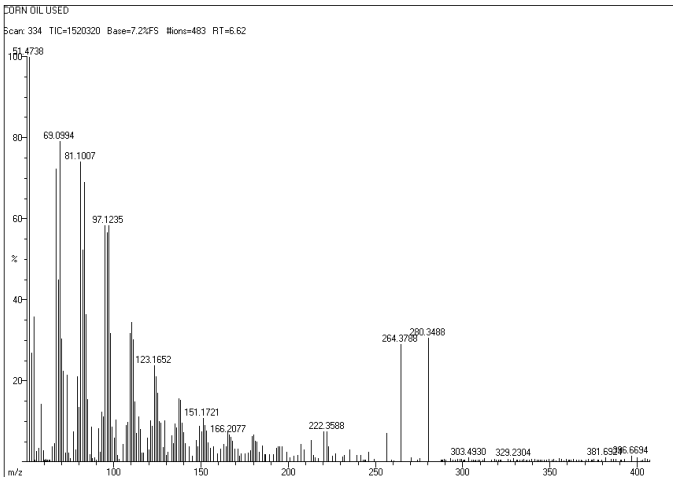

Fig 9 Mass spectrum of stigma sitosterol