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Research Article

The Effect of Repeated Frying on Antioxidants in Oil by using High Performance Liquid Chromatography

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ABSTRACT

In daily routine homemakers at home usually prefer to subsequent frying of oil in order to save money, but due to repeated frying, the antioxidants in oil degrades result in the oxidation of oil and due to oxidation of oil concentration of polar compounds in oil increases and makes the oil unhealthy for consumption. Antioxidants are basically added in the edible oil to increase the shelf life of the oil. The shelf life and the stability of natural antioxidants are more as compared to synthetic antioxidants. Synthetic antioxidants are added into the oil because during refining process the natural antioxidants get removed from the oil.

Keywords: Oil; Antioxidants; Synthetic; Hydrolysis; Oxidation

INTRODUCTION

Antioxidants itself defines its meaning, against oxidations or the molecules used to prevent or to reduce oxidation by series of chemical reaction. Their sole function is to scavenge the free radicals. It can be natural or synthetic.

Synthetic antioxidants are generally spike into the refined oil to prevent oxidation because during the synthesis of refined oil natural antioxidants are washed away during refining; hence refined oils are more prone to oxidation or rancidity therefore spiked with synthetic antioxidants. Synthetic antioxidants that are added:

- Tertiary Butyl Hydroquinone (TBHQ)
- Butylated Hydroxy Anisole (BHA)
- Butylated Hydroxy Toluene (BHT)
- Ethyl Gallet (EG)
- Propyl Gallet (PG)
- Dodecyl Gallet (DG)
- Octyl Gallet (OG)

Among the above synthetic antioxidants most commonly added ones are TBHQ and BHT, the reasons are:

- Higher thermal stability as compare with the other synthetic antioxidants
- Higher rate of retention in the oil on repeated frying as compare with the other synthetic antioxidants
- Lower rate of degradation in the oil on repeated frying as compare with the other synthetic antioxidants

India is a country where we prefer to eat tasty food rather than eating healthy foods. To make the food tastier we prefer deep frying, frying enhanced the taste and aroma of food. By doing this not only causes the degradation of nutrients but also the antioxidants, hence rancidity or rate of oxidation of fat present in refined oil started.

As per FSSAI [1] consumable limit of synthetic antioxidants in oil should be between (40 ppm-200 ppm).

As the degradation of antioxidants occurs it leads to an increase in the rate of oxidation or rancidity in oil, because deep frying of oil provides the temperature and oxygen for rancidity or oxidation. In day-to-day life generally, we do not throw or discard the oil after first frying, rather than discarding it we prefer repeated frying of the same oil in order to save money and oil. After repeated frying the concentration of both natural and synthetic antioxidants decreases, the rate of oxidation and rancidity increases, and hence the concentration of polar compounds increases which makes the oil not consumable. In oxidation oil level unsaturation of lipids decreases and the level of saturation of lipids increases in the oil which makes the oil unhealthy for consumption.

Health risk associated with the consumption of oxidized oil

Weaken the antioxidants immune system of human. Responsible for chronic diseases such as coronary heart disease, hypertension, vascular inflammation, diabetes etc. Causes liver damage reduces

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serum and hepatic vitamin E. Effects is worse in women in postmenopausal. Increase oxidative stress.

When oils are heated or fried at the temperature above the boiling point (temperature above 150°C) of water leads to dehydration and oxidation i.e., rancidity of oil. This leads to the formation of free fatty acids or free radicals or polar compounds. It is a myth that unsaturated fatty acid is good for consumption, they are more prone to oxidation due to higher degree of unsaturation in them. Oil with more linoleic fatty acids is more prone to oxidation.

Chemical reaction occurs during deep fat frying

Hydrolysis of oil: Hydrolysis means breakdown of molecule in presence of or with the help of water. Whenever food items fried in the oil leads to formation of water vapors (steam), these steam, oxygen, water started the chemical reaction in the oil. This reaction of hydrolysis basically occurs in the oil phase and in the interphase of oil and water. Rate of hydrolysis is more in shot, unsaturated fatty acids as compared to long, saturated fatty acids.

Oxidation of oil: Basically, two types of oxidation occur in oil:

- Thermal oxidation
- Autoxidation

Rate of thermal oxidation is more in oil as compare to the autoxidation. In oxidation water being weak nucleophile attacks at the ester bond in fatty acid and releases free fatty acids in presence of oxygen. There are three steps in oxidation of oil *viz.* initiation, propagation and termination.

Polymerization of oil: Polymerization of oil occurs when it is fried with some food. During frying volatile components (responsible for the aroma and flavor in the oil) degrades into non-volatile polar compounds, which is a polymer and as the concentration of polar compounds increases in the oil it becomes less consumable. The polymers formed by the decomposition of can be cyclic and acyclic.

Tertiary Butyl Hydroquinone (TBHQ)

It is a phenolic compound, synthetic antioxidant derived from hydroquinone used as a preservative in edible unsaturated oil. As per European Food Safety Authority (EFSA), Food and Drug Administration (FDA) and Food Safety and Standard Authority of India (FSSAI) the consumable limit of TBHQ is 40 ppm-200 ppm. It has been tested and found that within consumable limit it is non-toxic and non-carcinogenic (Figure 1).

In foods, TBHQ is used as a preservative for unsaturated vegetable oils and many edible animal fats. It does not cause discoloration even in the presence of iron, and does not change the flavor or odor of the material to which it is added. It can be combined with other preservatives such as Butylated Hydroxy Anisole (BHA). As a food additive, its E number is E319. It is added to a wide range of foods, with the highest limit (1 gram/kg) permitted for frozen fish and fish products. Its primary advantage is extending storage life.

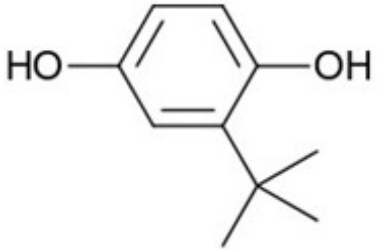

Figure 1: Structure of TBHQ.

LITERATURE REVIEW

According to Bahruddin Saad et al, Determined synthetic phenolic antioxidants in thirty-eight food samples comprised of oil, bread, butter, and cheese using reversed-phase HPLC. Their objective was to evaluate simple and efficient method for the quantification of phenolic antioxidants using HPLC. The instrument conditions they used were, C18 as stationary phase, mobile phase used was acetonitrile and water in 1:1 ration (v/v) with 1% acetic acid, UV detector. Synthetic phenolic antioxidants were extracted and run in HPLC. The antioxidants were found in all samples [2].

According to Qing guo et al, they studied the effect of repeated frying on the natural antioxidants in palm oil, they found on repeated frying the concentration of natural antioxidants decreases and concentration of polar compound increases like peroxides and free fatty acids. They also studied the stability of rosemary ethanol extract on repeated frying when used as antioxidants, they found that the rosemary ethanol extract was more stable on frying as compare to the natural antioxidants in palm oil, they also found the concentration of polar compounds decreased when rosemary ethanol extract used as an antioxidant on frying and it was equally efficient as compare to the synthetic antioxidants but safer in use as compare to the synthetic antioxidants [3].

According to Alam and Mohammad they studied the thermal stability and optimum concentration of the natural, synthetic and combination of natural and synthetic antioxidants in food processing. The effectiveness of these combinations was assessed rancimat test and they concluded that during high temperature treatment the combination of these natural and synthetic antioxidants leads to the either positive or negative synergism based on the type and concentration of individual antioxidants present in the combination [4].

According to Andrikopolous NK et al, they studied the repeated frying of virgin olive oil and sunflower oil and effect of repeated frying on retention of natural antioxidants. The natural antioxidants they studied was alpha, beta and gamma-tocopherol. They did the eight repeated frying in the same oil, they fried potato every time and found that 85% to 90% of alpha-tocopherol was retained after first frying and 15% to 30% after eighth frying whereas the beta and gamma-tocopherol completely disappeared after sixth frying [5].

According to Servili et al, they found that the phenolic compounds are the major antioxidants in the olive oil and they also studied the concentration of phenolic antioxidants in olive fruit, olive oil and virgin olive oil. They extracted the phenolic antioxidants from the sample and found that the concentration of phenolic antioxidants was maximum in olive fruit than in virgin olive oil than in olive oil. The antioxidants were lost during the process of extraction of oil from fruit and difference in the concentration of phenolic antioxidants is due to the difference in the extraction processes. The instrument conditions they used were, C18 as stationary phase, mobile phase used was acetonitrile and water in 1:1 ration (v/v) with 1% orthophosphoric acid and 1% acetic acid in gradient elution, PDA detector. The phenolic antioxidants were extracted and run in HPLC [6].

According to Y. B. Che Man they studied the effect on antioxidants by changing the characteristics of oil, those characteristics they preferred were refined, bleached and decolorized RBD palm oil. They evaluated various synthetic antioxidants such as TBHQ, BHA, and BHT and found that the on-frying potato the concentration of BHA and BHT decreases more significantly as compare to TBHQ.

This order of decreasing is same in all three oils but concentration decreases more in bleached oil than decolorized than refined oil [7].

According to Kuniko Miyagawa et al. They studied the retention of tocopherol by frying coated potato, they studied for all three isomers of tocopherol those are alpha, gamma and delta. They found that the retention time of alpha tocopherol is more as compare to the gamma and delta tocopherol, and also found that delta with least retention time. The method they used was fluorescence spectroscopy [8].

According to Barrera Arellano.D et al., They took the oil samples with different in their degrees of unsaturation namely, Soyabean oil, Olive oil, Palm Olein oil and Sunflower oil and studied the retention time for both natural and synthetic antioxidants. For natural antioxidants they studied for tocopherol and for the synthetic antioxidants they studied phenolic antioxidants. They fried potatoes in the oils and found that the rate of retention of natural antioxidants were more as compared to the synthetic antioxidants and rate of degradation of antioxidants were more in oil containing high linoleic fatty acid which was more in sunflower oil hence, they concluded that it is not good for health for the consumption of repeated fried oil containing more of linoleic fatty acid such as sunflower oil [9].

According to Goswami et al., They reported that continuous consumption of repeated fried refined oil leads to some health disorder due malfunctioning of antioxidants immune system which then leads to some chronic disorder such as diabetes, coronary heart disease, hypertension. Increased concentration of polar compounds and free radicals leads to oxidative stresss [10].

According to Francisco Visioli et al., They found that the people in Mediterranean basin are less prone to coronary heart disease and cancer due to their daily habit of cooking food in olive oil and vine oil. Olive and vine have a antioxidants defense mechanism against environment, they have phenolic class of antioxidants, these are more stable class of antioxidant than other antioxidants. The oil they prefer to eat is virgin oil, virgin oils prepare directly by pressing the fruits. This increases the shelf life of oil [11].

According to Andrikopolous et al., Phenolic compounds are of great importance as natural antioxidants of vegetable oils. Olive oil has been a very important product in the Mediterranean Countries for centuries, and in Greece the consumption of olive oil comprises over 50% of all edible oils consumed [12].

According to Aladedunye and Matthaus, Lipid oxidation is one of the major problems during the process of frying and storage, resulting in the generation of free radicals as well as degradation of food quality and possibly leading to diseases ultimately [13].

According to Casarotti and Jorge, in order to retard, reduce or prevent oxidative deterioration, antioxidants are added to oil, such as Butylated Hydroxy Anisole (BHA), Butylated Hydroxytoluene (BHT), Propyl Gallate (PG) and Tertiary Butyl Hydroxyquinone (TBHQ) [14].

According to Nenadis et al., synthetic antioxidants are effective at low temperatures. However, during frying, the oil temperature is around 180°C, the heat of which can quickly destroy antioxidants, for they are erratic and can easily evaporate at high temperatures, which may be related to their structures. Therefore, they cannot provide the oil with enough protection against oxidative deterioration [15].

Chammem et al., Natural antioxidants mostly come from spices and herbs, which are known to contain many phytochemicals that can slow down lipid oxidation. Among the plant extracts that have been studied, rosemary extract shows a remarkable antioxidant effect and it is likely to be used industrially.

Shahidi et al, Antioxidants were found to protect lipids against oxidation either by quenching free radicals or scavenging oxygen, among others. Antioxidants are substances that, when present in foods at low concentrations compared with that of an oxidizable substrate, markedly delay or prevent the oxidation of the substrate. Antioxidants that fit in this definition include free radical scavengers, inactivators of peroxides, and other Reactive Oxygen Species (ROS), chelators of metals, and quenchers of secondary lipid oxidation products that produce rancid odors.

H. H. Hussain et al, Scientists are attempting to develop novel synthetic antioxidants aimed at retarding the effects of free-radical-induced damage in various food products as well as in the human body cells. Synthetic antioxidants used in the food industry can be added as direct additives or indirectly through diffusion from packaging material.

INSTRUMENTATION

Chromatography

A technique for the separation of a mixture by passing it in solution or suspension through a medium in which the components move at different rates. The mixture is dissolved in a fluid called the mobile phase, which carries it through a structure holding another material called the stationary phase. The various constituents of the mixture travel at different speeds, causing them to separate. The separation is based on differential partitioning between the mobile and stationary phases.

There are mainly six types of chromatographic techniques used in chemistry:

- Paper Chromatography
- Thin Layer Chromatography (TLC)
- Liquid Chromatography (LC)
- High Performance Liquid Chromatography (HPLC)
- Ion Chromatography (IC)
- Gas Chromatography (GC)

High Pressure Liquid Chromatography (HPLC): High performance liquid Chromatography is a form of column chromatography that pumps a sample mixture or analyte a solvent at a high pressure through a column with chromatographic packing material. HPLC has the ability to separate, and identify compounds that are present in any sample that can be dissolved in a liquid in trace concentrations as low as parts per trillion. Because of this versatility, HPLC is used in a variety of industrial and scientific applications, such as pharmaceutical, environmental, forensics, and chemicals.

The advantage of HPLC over other forms of liquid chromatography are; The HPLC column can be used many times without regeneration, the resolution achieved on such column far exceed that of the older methods, analysis times are generally much shorter, the instrumentation of HPLC leads itself to automation and quantitation. The new HPLC instruments designed could generate up to 6,000 psi (400 bar) pressure.

Normal phase: Chromatography, involves the use of a polar stationary phase (e.g., silica or alumina) and a non- polar mobile phase (e.g. hexane, chloroform, methanol, ACN, methylene chloride and mixtures of these). Here, polar analyte is retained more strongly by the column and thus allowing the elution of non-polar constituents first. Adsorption strengths between the column and the analytes increases with increased the polarity of the analyte, and thus the interaction between the polar analyte and the polar column increases the elution time or the time analyte to exit the column.

Reversed phase: (RP-HPLC/RPC) involves the non-polar stationary phase is used with the polar mobile phase. The hydrocarbon bonded silica-based packing material is used in conjunction with an aqueous/organic mobile phase although pure aqueous (hydrophobic chromatography) and polar organic (non-aqueous reversed phase) eluents are also used. This is, because of its versatility and simplicity, by far the most popular technique contemporary used in HPLC. RP is based on the principal of hydrophobic interaction. RP-HPLC system is more commonly used than the normal phase chromatographic systems.

Operation: A reservoir holds the elution solvent (the mobile phase as it moves through the column). A high- pressure pump (solvent delivery system, as it facilitates the movement of the mobile phase, typically milliliters per min.

An injector (called the sample manager) introduces (inject) the sample into constantly flowing stream of the mobile phase that bears the sample into HPLC column. The injector could either be auto sampler (where it injects a particular volume of the sample automatically) or it could be a manual sampler (where an appropriate volume of the sample has to be introduced after each run). The column is filled with the chromatographic packing which performs the separation effectively. This material is known as the stationary phase because it is retained tightly by the column hardware. A detector is needed to identifies and analyses the separated components as they elute from the HPLC column. The mobile phase eventually exits detector and is then sent to the waste, or collected as per the requirement.

Instrumentation: Mobile phase delivery system; A glass reservoir is generally used to contain the mobile phase contents. The mobile phase or the elution solvent, in HPLC is usually a mixture of non-polar and polar organic liquids whose respective concentration depending on the nature and composition of the sample. The preference of the solvents depends on the nature of the operation mode, i.e. isocratic and gradient and also on the solubility of the sample in the chosen mobile phase. The solvent must be pure (including water). In choosing the mobile phase the following must be prioritized; a low viscosity, a suitable polarity, and proper eluting strength, compatibility with the detector in use, and finally, whether there is a need for removal of the solvent from the analytic, once the analysis has been done. The solvent must be always degassed before use in the system which can be done by the application of heat, vacuum, ultrasound or by purging a stream of inert gas like helium. The absence of a degassed solvent may lead to: formation of air bubbles in the leading to loss of solvent flow, formed bubbles at column outlet, eventually causing the release of high pressure thus leading to chromatogram disruption or baseline drift (Figure 2).

Stationary phase: The commonly used packing materials are silica, polysaccharide, styrene-divinyl benzene, alumina, as well as diatomaceous earth with silica gel being the most popular choice. It has been widely used because it helps in achieving a good separation due its pore size, surface properties and particle size. Silica dissolves at high pH the use of solvents that exceed pH 7 are not recommended. Additionally, the combination of high temperature along with extremes of pH can also contribute to the deterioration to silica. The stationary phase is usually made up of hydrophobic alkyl chains which interact with the analyte. Three common chain length are used: C4, C8 and C18 is preferred for proteins.

Pumps: The pumps used in HPLC system are controlled electronically to modulate the pressure and flow rates within close limits. Pumps vary in pressure capacity, but their performance is measured on their ability to yield a consistent and reproducible volumetric flow rate. Pressure may reach as high as 40 MPa (4000 psi), or about 400 atmospheres. Modern HPLC systems have been improved to work at much higher pressures. An HPLC pump is also called a "solvent delivery system." The purpose of the pump is to maintain a constant flow of mobile phase through the HPLC system. This is accomplished regardless of the back pressure caused by the flow resistance of the HPLC column. HPLC pumps are capable of pumping the following types of liquids: aqueous buffers, organic solvents, viscous fluids, aggressive fluids, radioactive liquids, brine solutions, and more.

Column: Columns are the main component in HPLC because the column is responsible for the separation of the sample components. The sample passes through the column with the mobile phase and separates in its components when it comes out from the

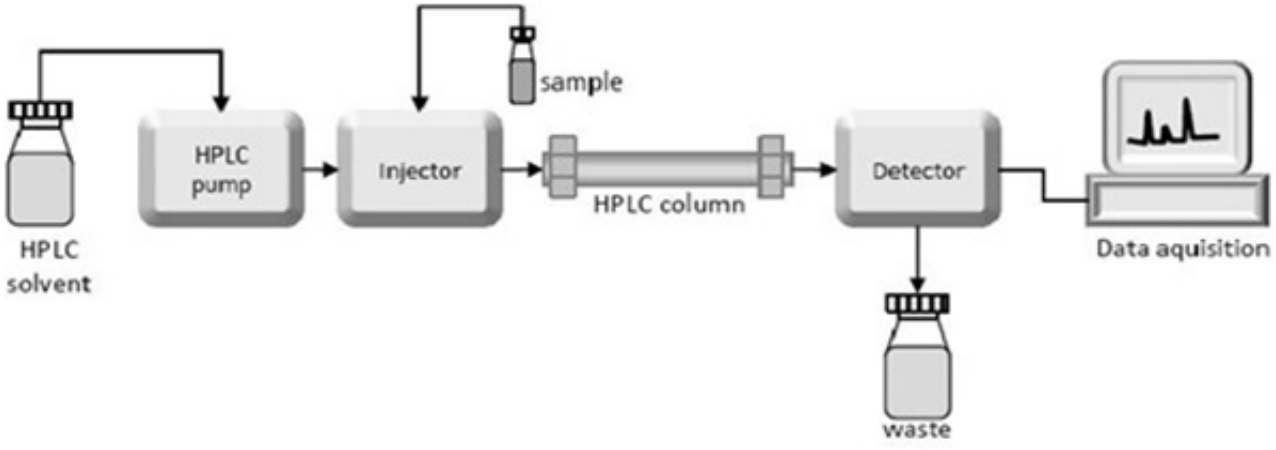

Figure 2: HPLC Instrumentation.

column. The material filled in the HPLC columns is known as a stationary phase. The columns are generally made of stainless steel. The columns involved in RP-HPLC systems are tubular and their dimensions have the following configuration internal diameter × length (e.g. 4.6 mm × 250 mm). Particle size and pore size the columns in general are packed with particles of silica. These particles are generally ordinarily characterized by their particle and pore size. Particle sizes range between 3 and 50 microns, with 5 µm particles being the most popular prominent choice. Larger particles tend to develop less system pressure whereas smaller particles as opposed will develop more pressure. The smaller sized particles give increased separation efficiencies.

Detectors: The detector is outlet end of the column where it receives the outcome of the column and monitors in real time. They are most sophisticated and expensive component of HPLC system. The detector converts a change in effluents into an electrical signal that is recorded by the data system.

Detectors are classified into:

1. **UV/VS Detector:** Measures the ability of solutes to absorb light at a particular wavelength in uv or vs wavelength range. when light of a certain wavelength is directed at a flow cell, the substance inside the flow cell absorbs the light. As a result, the intensity of the light that leaves the flow cell is less than that of the light that enters it. An absorbance detector measures the extent to which the light intensity decreases. (i.e., absorbance).
2. **Photo diode array detector:** This is operated by simultaneously monitoring absorbance of solutes at several different wavelengths. Light from the broad emission source such as deuterium lamp is collimated by achromatic lens system so that the total light passes through the detector cell on to a holographic grating. In this way, the sample is subjected to light of all wavelength generated by the lamp. The dispersed light from the grating is allowed to fall on to a diode array. The array may contain many hundreds of diodes and the output from each diode is regularly sampled by a computer and stored on a hard disc.
3. **Fluorescence detector:** It is near ideal detector for those solutes that exhibit molecular fluorescence. Their sensitivity depends on the fluorescence properties of the components in elute.
4. **Refractive index detector**: Also known as RID detector measures the molecules ability to deflect light in a flowing mobile phase in a flow cell relative to a static mobile phase contained in a reference cell. The amount of detection is proportional to the concentration of the solute in the mobile phase.

Data acquisition: Signal obtained from the detector can be collected on suitable chart recorders or appropriate electronic integrators which vary in their complexity and ability of processing the data, in their storage.

The computer integrates the response obtained from the detector to each component and places it accordingly on a chromatograph that is easy to read and interpret.

The output from the detector is recorded in the form of series of peak where each peak represents a compound present in the sample which passes through the detector. The area that lies under the peak will be proportional to the amount of substance that passes through the detector, and this area can be calculated automatically by the computer that is linked to the display.

MATERIALS AND METHODOLOGY

Apparatus:

- HPLC Instrument
- Weighing Balance
- Deep Freezer
- Micropipette
- Centrifuge
- Vortex
- Vacuum Filter Assembly
- Sonicater
- PVDF Syringe Filter 0.45 µm
- HPLC Vials
- Centrifuge Tube (15ml)

Reagents:

- Acetonitrile (HPLC Grade)
- Milli Q Water

Instrument Conditions:

- Column: C18 (250 × 4.5 mm,5 µm)
- Column Temperature: 35°C
- Wavelength (λ): 280 nm
- Detector: PDA
- Flow Rate: 0.6 ml/min
- Flow Type: Isocratic
- Injection Volume: 20 µl
- Run Time: 10 min
- Mobile Phase: Acetonitrile (0.98), Milli Q Water (0.02)
- Standard Diluent: Acetonitrile

Standard:

- Stock standard of all antioxidants were prepared in acetonitrile
- Working standard of 10 ppm and 20ppm mix were prepared in acetonitrile

Preparation of Standard: Working standard was prepared from already prepared stock solutions of TBHQ and BHA antioxidants and the diluent used was methanol.

Formula used for the working standard,

$C_1V_1 = C_2V_2$

Where,

C1- concentration of stock standard.

V1- volume taken from stock standard to make the desired concentration.

C2- concentration of working standard.

V2- volume in which working standard was made.

From the calculation the calculated volume of each antioxidants was taken in an Eppendorf and remaining diluent (ACN) was then

added to the Eppendorf and vortex to make 100 ppm mix from this 10 ppm mix and 20 ppm mix were made using above formula in a vial.

For 10 ppm mix-100 µl of standard from 100 ppm mix and 900 µl diluent (ACN) added into the vial.

For 20 ppm mix-200 µl of standard from 100 ppm mix and 800 µl diluent (ACN) added into the vial.

STANDARD AND SAMPLE CHROMATOGRAMS

Procedure for Sample Preparation

- Take 1 gm of sample in 15ml centrifuge tube
- Make up to 10 ml with diluent ACN
- Vortex for 10 min-15 min and shake till completely homogenized
- Sonicate for 5 min-10 min
- Keep centrifuge tube in deep freezer for 1hrs

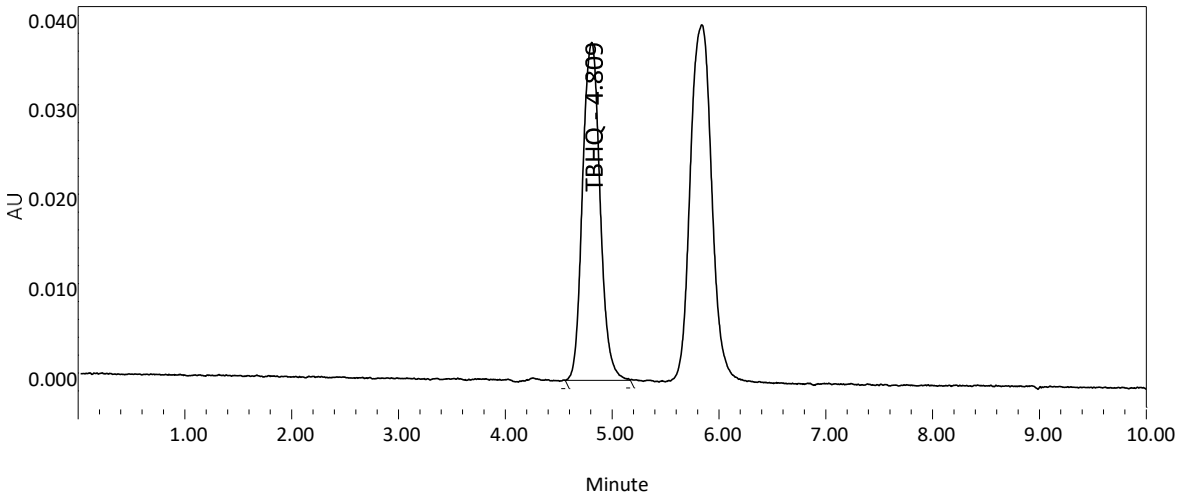

Figure 3: Standard TBHQ 10 ppm.

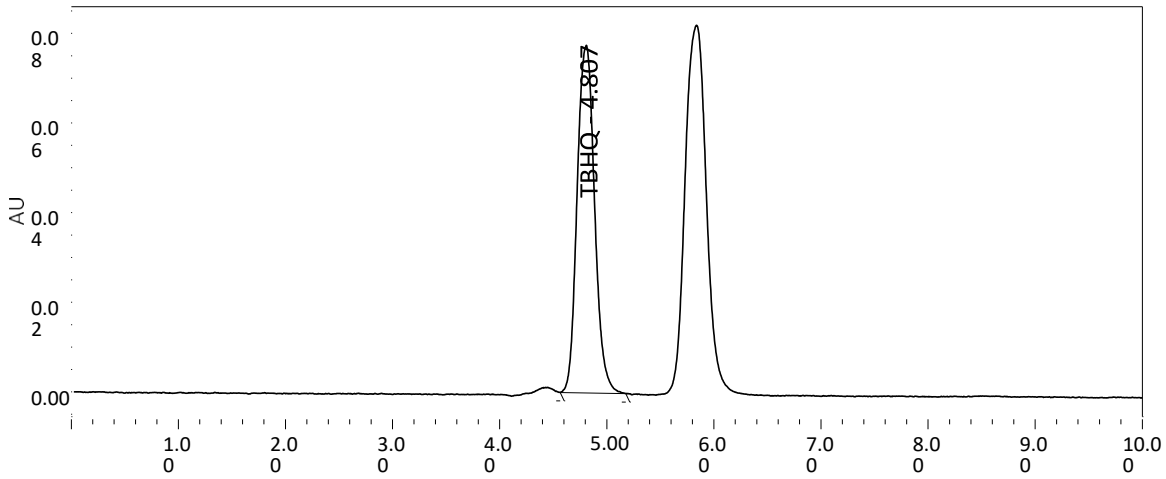

Figure 4: Standard TBHQ 20 ppm

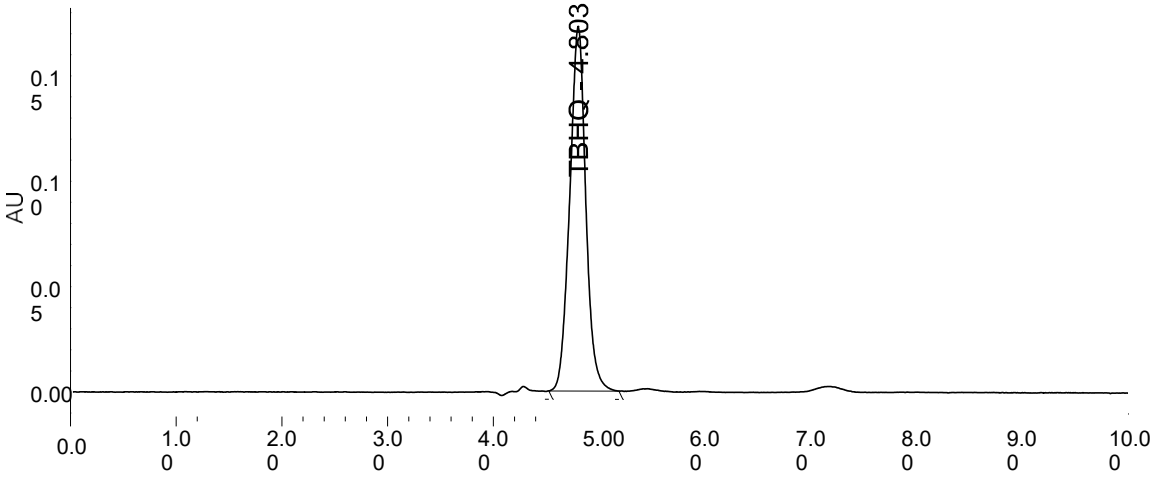

Figure 5: Sample Sunflower Oil (No Fry).

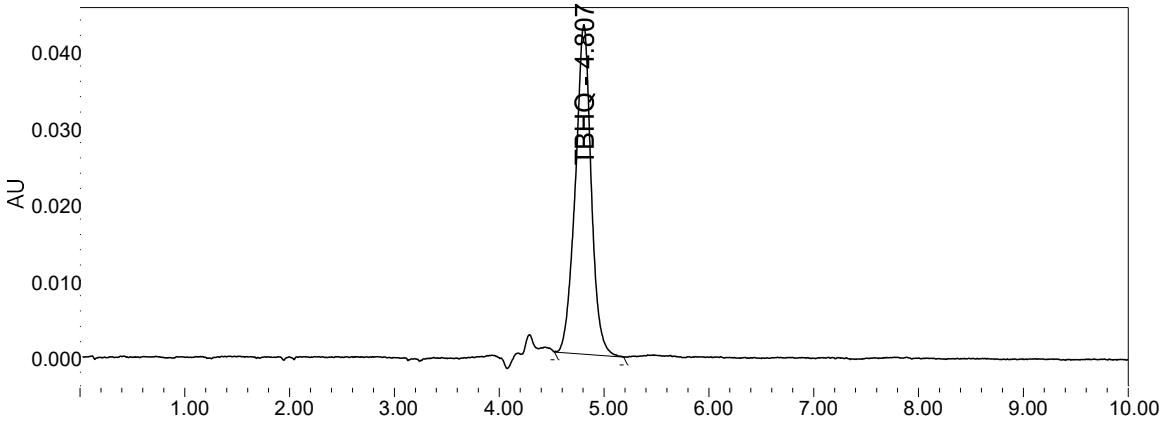

Figure 6: Sample Sunflower Oil [1st Fry]

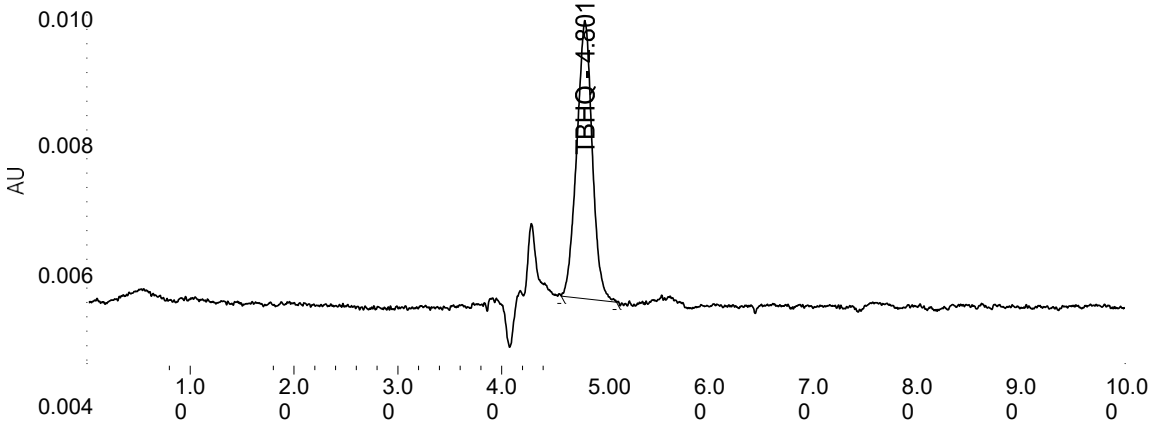

Figure 7: Sample Sunflower Oil (2nd Fry).

• Collect supernatant and filter in 2 ml HPLC vial through 0.45 µm syringe filter

• Run on HPLC under required set of condition (Figures 3-7).

RESULT AND DISCUSSION

Calculation

Concentration in ppm =

$$\frac{\text{Sample area} \times \text{Standard Concentration} \times \text{Volume Makeup}}{\text{Standard Area} \times \text{Sample Weight}}$$

I calculated the concentration of synthetic antioxidants in all samples and compile the result in tabulated form.

CONCLUSION

Nowadays it becomes a common practice of repeated frying of edible oil at home, but it's not a safe practice as frying mostly done in refined oils whose natural antioxidants remove from them while refining process, so to increase the shelf life of oil, these refined oils are spiked with synthetic antioxidants. Most commonly used antioxidants is Tertiary Butyl Hydroxy Quinone (TBHQ), but it is not effective at high temperature, high temperature and oxygen provide an optimum condition for the oxidation of unsaturated fatty acid present in the oil leads to accumulation of polar compounds and free fatty acid in oil and at such high temperature TBHQ degrades, hence is shouldn't be preferred to have repeated fried oil for consumption.

It was found from the experiment that the decrease in the concentration of TBHQ on repeated frying is more in case with sunflower oil from 189 ppm to 10 ppm. In No fry the concentration of TBHQ was 189 ppm, in 1st fry concentration of TBHQ was 125 ppm, in 2nd fry concentration was 70 ppm and completely degraded in 3rd fry.

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