SSRG International Journal of Applied Chemistry
ISSN: 2393 – 9133 / https://doi.org/10.14445/23939133/IJAC-V11I3P101

Volume 11 Issue 3, 1-14, Sep-Dec 2024

Original Article

Studying Phospholipids Present in Coconut Oil by Different Methods of Analysis

Pritha Nag

Ascend International School, Maharashtra, India.

Corresponding Author : prithaa.nag@gmail.com

Received: 02 September 2024 Revised: 04 October 2024 Accepted: 20 October 2024 Published: 05 November 2024

Abstract *- Over an extended period, phospholipids within a container of manufactured and processed coconut oil begin to settle and separate from the coconut oil, resulting in impurities within the oil. However, as phospholipids provide nutrients and are integral to coconut oil, removing them entirely is inefficient. Instead, understanding the quantity of phospholipids present in coconut oil can help to understand how much to remove without stripping the oil of its nutrients. This research paper delves into three separate methods of analysing the presence and quantity of phospholipids: Thin Layer Chromatography (TLC), High Performance Thing Layer Chromatography (HPTLC) and UV Spectrophotometer. The importance of this study relates to furthering the understanding of phospholipids in coconut oil, and by analysing three of the most popular analytical techniques, the pros and cons can be weighed and used accordingly. In this paper, TLC and HPTLC undertake a qualitative approach as they confirm the presence and quantity of phospholipids through observation and not measurement. UV spectrophotometer undertakes a quantitative approach as it confirms the presence and quantity through measurement. The research in this paper focuses on the workings of each approach, the challenges faced by each method, as well as when each method can prove to be most helpful. Thus, through rigorous investigation and trial and error, this work aims to understand how to quantity and confirm phospholipids. It serves as a foundation for creating purer coconut oil that retains all its essential elements.*

Keywords *- Chromatography, Fatty acids, HPTLC, TLC, UV Spectrophotometer.*

1. Introduction

Edible oils majorly consist of around 96% triglycerides along with other minor components like phospholipids, phytosterols, tocopherols, etc. Generally, the composition will remain the same. However, the composition will vary depending on what type of edible oil is being observed. [7] A major part of edible oils are triglycerides, which are composed of two main components: glycerol backbone and three fatty acid chains.

The glycerol backbone ($C3H8O3$) is a simple sugar alcohol compound that consists of a three-carbon chain, where each carbon is connected to a hydroxyl group (-OH) at the end of the chain. [11] The glycerol backbone's main function is to act as a backbone to the three fatty acids. [8] The saturation will vary depending on the fatty acid and the chain length. There are three types of fatty acids: Monounsaturated Fatty Acids (MUFA), Polyunsaturated Fatty Acids (PUFA) and Saturated Fatty Acids (SFA).

As saturated fatty acids have no double bonds, they have a straighter and more linear structure. Monosaturated fatty acids contain one double bond, which creates a slight kink in the molecule. In contrast, polyunsaturated fatty acids have two or more double bonds, which create multiple kinks within the molecule. Triglycerides are formed through an esterification reaction, and during this formation, water molecules are released as glycerol binds with the three fatty acids (SFA / MUFA / PUFA). When glycerol binds with the three fatty acids, it can be any combination of the three fatty acids, and it is relative to that specific triglyceride and edible oil.

The types and combinations of the three fatty acids combined with the three-glycerol backbone determine the physical and nutritional properties of the triglyceride. [10] The minor components of edible oil are sterols, tocopherol, tocotrienols, phospholipids, phenolics, and many more.

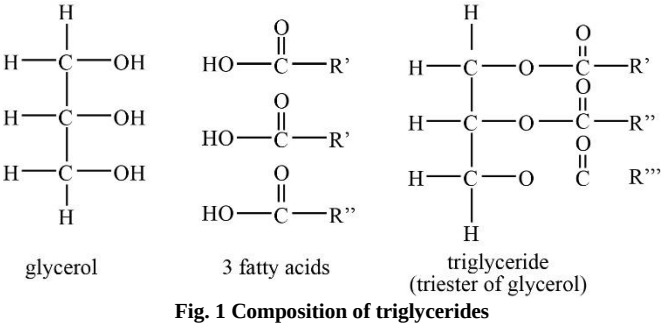


Fig. 1 Composition of triglycerides

Table 1. List of each major oil and its key properties

	Sunflower Oil	Peanut Oil	Mustard Oil	Coconut Oil	Palm Oil
Fatty Acids (major)	Linoleic acid Oleic acid	Oleic acid Linoleic Acid	Erucic Acid Oleic Acid	Lauric Acid	Palmitic acid Oleic acid
Melting Point (°C)	-17°	3°	-18°	24°	35°
Viscosity	Relatively low	Moderate	High	Solid at room temp, low viscosity when melted (to liquid)	Moderate to high viscosity
Refractive Index (at 40°)	1.465-1.469	1.460 – 1.465	1.464 – 1.4760	1.448 – 1.450	1.454 – 1.456
Density (at 20°)	0.920-0.924	0.910 – 0.915	0.910 – 0.920	0.924 – 0.927	0.890 – 0.920

This report focuses only on coconut oil. Further details of the coconut oils are mentioned in subsequent sections.

2. Phospholipids in Coconut Oil

2.1. Structure and Properties of Coconuts

Coconut oil is viscous (depending on temperature), dense, not soluble in polar solvents and does not have a high refractive index (placed at around 1.43 – 1.46). As coconut oil has high levels of saturated fat, it should have a higher melting point. However, because coconut oil has shorter carbon chains packed less efficiently and significantly weaker intermolecular forces, they do not have a high melting point. Along with this, the presence of lauric acids causes a lower melting point.[6] The shell that surrounds the kernel of the coconut is known as the endocarp/shell, and it is a hard spherical covering that is around 3-5 mm thick. It is mostly used as fuel in copra production, though other products like charcoal, activated carbon and glues can be created and extracted from the endocarp. The husk/mesocarp is around 5-10 cm thick and fit to cover the shell. Similarly to the shell, it is used for fuel in farms and products like activated carbon, rope, and geotextiles. The copra is the dried section of the meat in the coconut, and it acts as the kernel of the coconut. Fresh kernels have around 50% moisture, and there are a variety of drying techniques to remove the moisture. Once the drying process is complete, the moisture content will be reduced to around 6-8%. Initially, kernels are found in coconut. However, once the coconut is dried and the moisture content is reduced, the kernel becomes a copra. This conversion from kernel to copra is vital to draw out oil by the traditional mechanical extraction. [6]

2.2. Classes of Phospholipids

Phospholipids are a group of lipids that contain a hydrophilic head and hydrophobic tail. Phospholipids are identified through their hydrophilic head and hydrophobic tail, and by having these, they exhibit their amphiphilic qualities and dictate the properties of that specific phospholipid. Thus, according to the head and tail of the phospholipid, each phospholipid's traits would vary. [12] Phospholipids (PL) are present in multiple sources, including foods with high content of PLs naturally. Some are meat, egg yolk, guts, vegetable oil, brain, etc. [7] There are two classes of phospholipids: glycerophospholipids and sphingophospholipids. This classification is based mainly on the type of alcohol backbone. Within these two classes, phospholipids have different properties but will have the same alcohol backbone. [7]

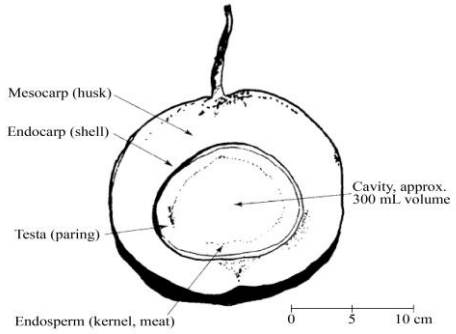


Fig. 2 Mature coconut labelled

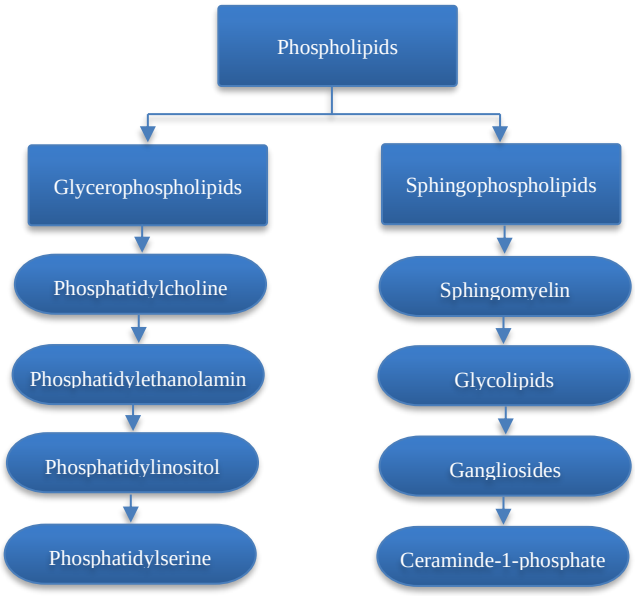


Fig. 3 Classification of phospholipids

2.2.1. Glycerophospholipids

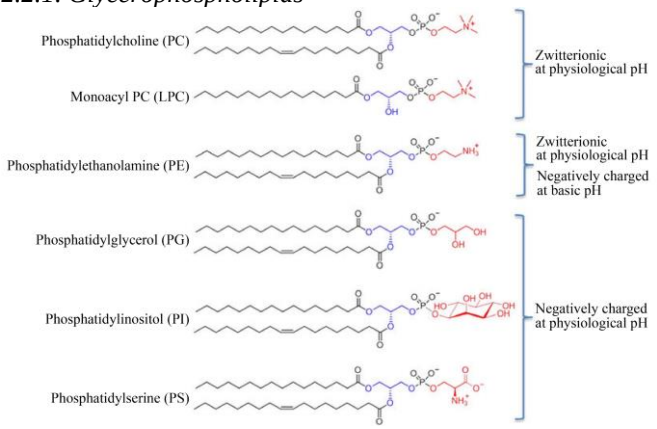


Fig. 4 Types of glycerophospholipids

Glycerophospholipids (GPLs) are unsaturated fatty diglycerides that contain a phosphatidyl ester linked to their terminal carbon. They represent the most prevalent class of phospholipids. Glycerophospholipids comprise a phosphate group, two fatty acids, a glycerol backbone and a polar head that dictates the type of glycerophospholipid, which can be varied. They have a specific type of molecular structure that allows glycerophospholipids to form their lipid bilayers critical to cell membrane function. The glycerol molecule forms the backbone, which has three carbon atoms. Each carbon atom (in this backbone) has a hydroxyl group (OH) that forms bonds with other molecules. Two hydroxyl groups on glycerol are esterified using fatty acids and can be esterified to fatty acid chains of glycerol. With the help of a fatty acid's carboxyl group (COOH), hydroxyl groups can form ester bonds. The fatty acid chains usually measure out to around 14-24 carbons. They can differ in the number of double bonds that are saturated or unsaturated, which will impact the fluidity and function of the lipid bilayer. The molecule possesses hydrophilic properties (water-attracting) because the phosphate group is attached to a third hydroxyl group of glycerol, the main feature of all phospholipids. The phosphate group is attached to another molecule, the polar head group. This head group can differ from different types of glycerophospholipids. [7]

2.2.2. Sphingophospholipids

Phospholipids, known as sphingosophospholipids, depend on the 18-carbon sphingosine backbone of amino acids. Rather than a glycerol backbone, they have a sphingosine backbone, consisting of a fatty acid connected by an amide bond and a phosphate group usually linked to choline. This sphingosine backbone is an 18-carbon amino alcohol with a long hydrocarbon chain, which contains the amino group (NH2) and two hydroxyl groups (OH). A fatty acid is attached to this amino group of sphingosines through an amide bond, and this fatty acid is generally a long-chain fatty acid. As this fatty acid is linked to sphingosine, it forms a molecule known as ceramide, an integral part of the sphingolipids. The primary hydroxyl group of sphingosines can be phosphorylated, which is a chemical process of adding a phosphate group to an organic compound, which results in the formation of a phosphate group attached to the sphingosine backbone, and this phosphate group can be linked to various other head groups, to form various forms of sphingophospholipids. The most common types of sphingophospholipids are sphingomyelin, glycolipids, and gangliosides. [7]

2.3. Structure of Phospholipids

Each phospholipid is made up of key components that make up a phospholipid. These key components include glycerol backbone, polar head group, phosphate group and fatty acids. Breaking these up can deepen the understanding of the function of phospholipids.

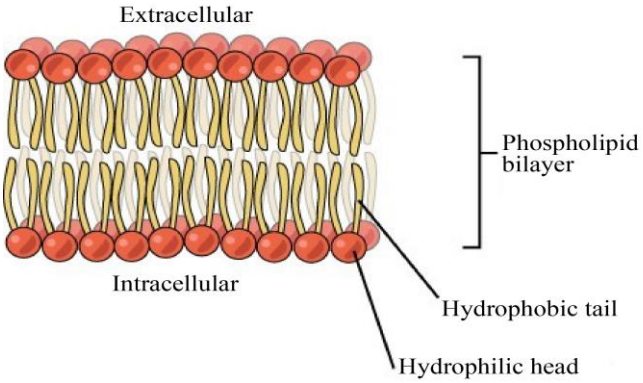


Fig. Glycerol backbone

2.3.1. Glycerol

Glycerol is a three-carbon molecule, with each carbon atom containing a hydroxyl group connected to it. This structure is the backbone for the rest of the phospholipid to be built upon. The glycerol backbone connects the hydrophobic fatty acid tails and the hydrophilic phosphate group, creating the phospholipids' amphipathic nature. [1]

2.3.2. Fatty Acid Chains

Two fatty chains are esterified to the glycerol molecule's first and second hydroxyl groups. A dehydration reaction between the carboxyl groups of fatty acids and the hydroxyl groups of glycerol creates the ester bonds.

Long hydrocarbon chains that are hydrophobic makeup fatty acid chains; the quantity and length of double bonds in these chains can differ. This hydrophobic tail helps to form the inner bilayer in the phospholipid, which creates the barrier to the passage of water-soluble substances. [7]

2.3.3. Phosphate Group

A phosphoester bond is formed by bonding a phosphate group to the third hydroxyl group of the glycerol molecule. This bond is created when phosphoric acid's hydroxyl group

combines with hydroxyl groups on other molecules to generate ester bonds. The phosphate group is negatively charged and hydrophilic on both sides of the membrane. The phosphate group is integral for the interaction between phospholipid and water, allowing the formation of the bilayer structure. [7]

2.3.4. Polar Head Group

Different types of phospholipids can be created as the phosphate group can be bonded to various polar molecules. Depending on the polar molecule that is bonded to the phosphate group, there will be different heads for the phospholipids. These different heads can dictate the properties of the phospholipid and the properties of the edible oil. [7]

Table 2. Varying headgroups in common phospholipids

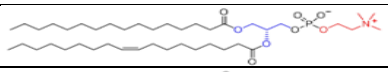
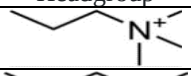
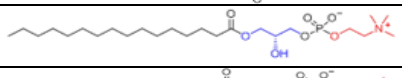
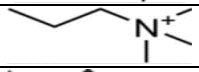
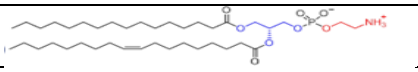
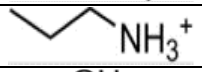
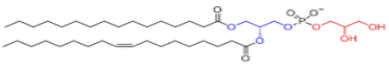
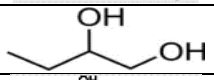
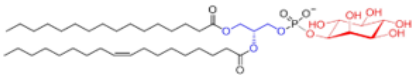
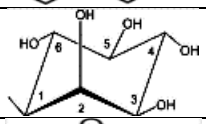
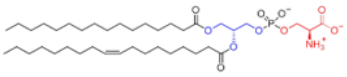
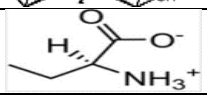
Phospholipid Name	Abbreviated Name	Phospholipid Structure	Phospholipid Headgroup
Phosphatidylcholine	PC		
Monoacyl Phosphatidylcholine	LPC		
Phosphatidylethanolamine	PE		
Phosphatidylglycerol	PG		
Phosphatidylinositol	PI		
Phosphatidylserine	PS		

Table 3. Common vegetable oils and their breakdown of phospholipids

Oil	Major Phospholipid	Minor Phospholipid
Sunflower Oil	Phosphatidylcholine (PC)	Phosphatidylethanolamine (PE)
Peanut Oil	Phosphatidylethanolamine (PE)	Phosphatidylethanolamine (PE)
Mustard Oil	Phosphatidylcholine (PC)	Phosphatidylethanolamine (PE)
Coconut Oil	Coconut oil is primarily saturated fatty acids in the form of triglycerides	Phosphatidylcholine (PC)
Palm Oil	Phosphatidylcholine (PC)	Phosphatidylethanolamine (PE)

2.4. Characterisation

Coconut is one of the most popular fruits from which oil can be extracted, either wet or dry. Before oil extraction, coconut oil phospholipids originate from the coconut endosperm (meat). [9] As the coconut grows and develops, the endosperm synthesises various lipids, including triglycerides and phospholipids. This is a naturally occurring process in the cells of the endosperm. Within the cells of the endosperm, the phospholipids are fused in the Endoplasmic Reticulum (ER) and the Golgi apparatus through enzymatic pathways. These synthesised phospholipids are integrated into the cellular membranes and the oil bodies of the coconut meat, and these oil bodies are small lipid droplets surrounded by a stabilizing layer known as a phospholipid monolayer. [8] Coconut phospholipids largely consist of phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylcholine (PC).

2.5. Degumming and Extraction of Phospholipids

Phospholipids end up in coconut oil during the extraction process. In either extraction method (dry or wet), the solid components are separated from the coconut meat oil. Some phospholipids in the cellular membranes and oil bodies can be transferred over. This happens because the mixture (not coconut oil yet) contains oil, water, and solid residues after the dry or wet process. As the oil is less dense than the two, it separates from the water and solids by floating to the top. The phospholipids are part of the oil droplets' surface and thus get carried out into the oil phase. The oil obtained after the separation is known as crude oil. Coconut crude oil will contain tiny quantities of phospholipids, large amounts of triglycerides, and minor components like free fatty acids. [12] During the refinement process, the crude oil undergoes something known as degumming. Degumming is a key step in refining edible oils. The purpose of degumming is primarily to remove impurities, like phospholipids, to ensure purer oil. It also allows for a longer and more stable shelf life, prevents processing problems, and improves the oil quality. Water will be added to the crude oil, which will cause the phospholipids to hydrate and combine, forming gums. These hydrated gums are significantly more polar than the oil and can thus be easily

separated from the oil through centrifugation or settling. [1] An acid like citric acid or phosphoric acid will be incorporated into the crude oil, which helps to separate the non-hydratable phospholipids and helps the phospholipids turn into hydratable phospholipids. Water is then added, which will help separate the hydratable phospholipids from the oil. Lastly enzymes will be added to the crude oil (e.g. Phospholipases) to break down any remaining phospholipids into more water-soluble components. [2]

2.6. Composition

As coconut oil is primarily made of triglycerides and other lipid components, the composition of phospholipids is significantly smaller than in other edible oils. However, these minor phospholipids help contribute to significant properties of coconut oil that can be beneficial. [5]

2.6.1. Phosphatidylcholine (PC)

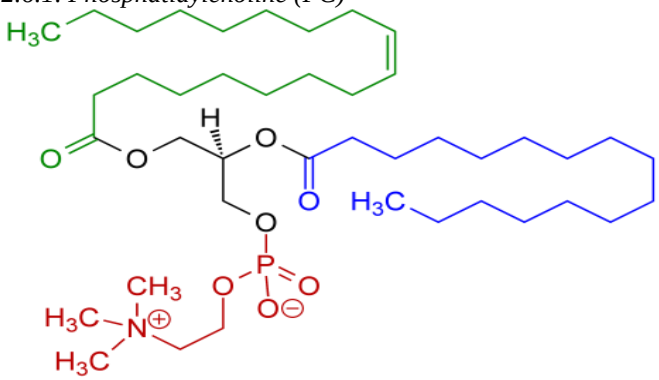


Fig. 6 Phosphatidylcholine

PC consists of a choline head group, phosphate group, glycerol backbone and two fatty acid chains. PC brings emulsifying properties because of its amphiphilic nature. It means that PC can stabilise the emulsions between oil and water. [7]

2.6.2. Phosphatidylethanolamine (PE)

PE and PC share similar structures; however, PE has an ethanolamine head group, creating different properties between phospholipids. It also acts as a natural emulsifier, contributing to the oil's nutritional profile. It is a significant part of the cell membranes and contributes to the structural integrity and composition of the lipid bilayer. This is vital for maintaining the barrier function of membranes, which is necessary to separate and protect cells from their surroundings. PE is also what helps to ensure the fluidity of the cell membranes. The PE influences the packing and organisation of the lipid molecules within the membrane. [7]

2.7. Research Problem and Objectives

Over an extended period, phospholipids within a container of manufactured and processed coconut oil begin to settle and separate from the coconut oil, resulting in impurities within the oil. However, as phospholipids provide nutrients and are integral to coconut oil, removing them entirely is inefficient. Instead, understanding the quantity of phospholipids present in coconut oil can help to understand how much to remove without stripping the oil of its nutrients. This research paper delves into three separate methods of analysing the presence and quantity of phospholipids: Thin Layer Chromatography (TLC), High Performance Thing Layer Chromatography (HPTLC) and UV Spectrophotometer. The importance of this study relates to furthering the understanding of phospholipids in coconut oil. The pros and cons can be weighed and used by analysing three of the most popular analytical techniques. Past studies have either focused only on the presence of phospholipids in oils or evaluated specific techniques. However, these techniques do not weigh the strengths or limitations of each method regarding the extraction of phospholipids. This paper addresses the absence of a systematic comparison of the qualitative and quantitative methods used to analyse phospholipids in coconut oil. The research aims to resolve this issue by conducting a rigorous investigation into the three different analytical techniques (as observed in the later sections of the paper), assessing their effectiveness and identifying how they can be used for a specific problem or practice. This investigation helps to create a framework for allowing coconut oil to maintain and preserve its essential nutrients.

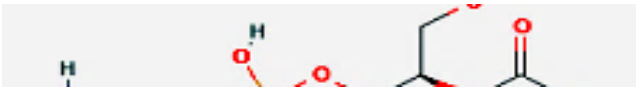


Fig. 7 Phosphatidylethanolamine

3. Materials and Methods

3.1. Extraction of Phospholipids in CNO

To carry out any analysis of phospholipids, phospholipids must first be extracted from coconut oil. Refer to the methodology below as the most efficient method of extracting phospholipids from coconut oil without having too much excess oil and maximum phospholipids.

3.1.1. Materials

Table 4. Materials for extraction of phospholipids from CNO

Material:	Quantity:
Beaker (150 mL)	1
Coconut Oil	80 mL
Water Bath	1
0.1% Orthophosphoric Acid (85%)	20 mL
Magnetic Needle	1
Stopwatch	1
Centrifuge Tubes (50 mL)	3
Centrifuge Machine	1
Dropper	2
Hexane	50 mL
Methanol	5 mL
Chloroform	5 mL
Acetone	2 mL
Eppendorf Test Tubes	2-3

3.1.2. Safety Considerations

- Ensure the need to wear goggles, gloves, a mask, and a lab coat throughout this experiment, and use correct PPE.
- Practice caution when using beakers as they are made from glass.
- Handle orthophosphoric acid, hexane, methanol, acetone and chloroform in a well-ventilated area or under a fume hood to avoid inhaling fumes.
- Only use the centrifuge machine with a lab assistant or someone who knows the machine.

3.1.3. Method

1. In a beaker, measure 80 mL of coconut oil.
2. After stirring the coconut oil, put it in a water bath at 50°C for 30 minutes.
3. After 30 minutes, in that same beaker, add 0.1% orthophosphoric acid (85%).
4. Put a magnetic needle inside the beaker and place it in a water bath heated at 60°C equipped with a magnetic stirrer. Ensure the magnetic stirrer is stirring the mixture.
5. Allow this to mix at 60°C for 30 minutes.
6. Take 3 centrifuge tubes and pour 30 mL into each tube.
7. Place the tubes into the centrifuge machine for 30 minutes at 25°C at a rate of 5000 RPM (rotations per minute).
8. After 30 minutes, remove the tubes, ensuring they stand upright and do not get mixed.
   Note: A clear distinction between oil and water and some white phospholipids around the tube should be observed.
9. Begin to remove the oil using a dropper.
10. Once a large quantity of the oil has been removed, pour around 25 mL of hexane into the tube.
11. Screw the lid to the tube and mix the hexane around, washing the phospholipids with hexane. The hexane will remove any excess oil without removing phospholipids.
12. Repeat this step one more time.
13. Discard the hexane.
14. Next, 5 mL of methanol was poured into the tube.
15. Cap the lid and swirl the tube, allowing the methanol to capture all the phospholipids.
16. Pour 5 mL of chloroform into the tube and swirl as done previously.
17. Add in 2 mL of acetone.
18. Swirl it around in the tube.
19. Small quantities of chloroform, methanol, and acetone are added using a dropper to get the most precise solution possible.
20. Transfer this into Eppendorf test tubes.
21. That will act as the phospholipid extract.

3.2. Thin Layer Chromatography (TLC)

TLC is commonly used to separate different elements of a mixture. TLC is conducted on a rectangular piece of aluminium paper coated with an absorbent material, and in this specific experiment, it is silica gel. Through TLC, each component of the mixture can be seen, as each component will leave a spot, showing distinctions between the composition of the mixture. TLC relies on the separation principle, which depends on the compounds' affinity to the stationary and mobile phases. Silica gel (TLC plate) acted as the stationary phase in this experiment. The TLC chamber was placed in glass, which was saturated using filter paper. The mobile phase was created with trial and error and references from various studies. Below is the methodology for finding the most accurate mobile phase that provides the best results. The compounds present in the mobile travel through the stationary phases at varying speeds based on their affinity for the stationary phase. Compounds in the mobile phase usually travel faster than the remaining compounds. The mixture constituent parts will present themselves as spots on the TLC plates at their appropriate locations, and this will confirm the presence of phospholipids in the coconut oil extract.

3.2.1. Materials

Table 5. Materials for TLC

Materials:	Quantity:
Beaker	2
Chloroform	75 mL
Methanol	25 mL
Ammonia (25%)	2 mL
TLC Chamber	1
Saturation paper/filter paper	1 sheet
Methanol	200 mL
Fridge	1
Copper Sulphate	20 g
Orthophosphoric Acid	8 mL
Sulfuric Acid	8 mL
Water Bath	1
TLC Paper	1 sheet
Cutter	1
Pencil	1
Ruler	1
Calliper	1-2
Tweezer	1
Aluminium Foil	1 roll
Hair Dryer (optional)	1
Oven	1
HPTLC dipping chamber	1

3.2.2. Safety Considerations

- Ensure the need to wear goggles, gloves, a mask, and a lab coat throughout this experiment, and use correct PPE.
- Handle chloroform, methanol, ammonia, sulfuric and orthophosphoric acid in a fume hood and avoid inhalation as they are toxic if ingested.
- Exercise caution when using the cutter.
- If using a hair dryer on the plate, wait 5 minutes, as the heat from the fire can cause the vapours from the plate to ignite.

3.2.3. Method

1. In a beaker, 75 ml chloroform, 25 ml methanol, and 2 ml ammonia were prepared (25%). Mix well. It will act as the mobile phase.
2. Cut a piece of saturation/filter paper to the desired size of the TLC chamber and place it inside to saturate it.
3. Pour 10 mL of the mobile phase into both sides of the chamber, ensuring that the saturation paper is also run by the mobile phase.
4. Take 200 mL of cooled methanol, 20 g of copper sulphate, and 8 mL of sulphuric acid and combine them all to make the derivatisation reagent in a water bath to ensure the mixture does not go higher than 20°C.
5. Once the derivatisation reagent has been created, pour it into an HPTLC dipping chamber and cover it with aluminium foil.
6. Cut a rectangular piece out of TLC paper (12x5 cm) using a cutter so the lines are exact.
7. Draw a pencil line around 1 cm above the bottom, and spot a dot in the centre of that line using that pencil.
8. Fill a calliper with the phospholipid extract (as created in section 3.2) along the pencil dot spot 5-6 drops.
9. After spotting each dot, allow the area to dry normally.
10. Once 5-6 dots have been spotted and dried, take a pair of tweezers and place the plate into the chamber as carefully as possible.
11. Ensure that it is placed as linearly as possible.
12. Once placed, cover the chamber with foil and then the lid.
13. Keep an eye on the plate, and when 0.5 cm of the plate is yet to be run by the mobile phase, take a pair of tweezers and remove the plate from the chamber.
14. Allow the plate to dry until the mobile phase appears entirely gone.
    Note: To speed up the drying process, use a hair dryer.
15. Once the plate is dry, take a pair of tweezers and carefully dip the paper in the derivatisation reagent for 2 seconds.
16. Take it out and allow it to dry for 1 minute.
17. Put it in an oven set to 150°C and let it dry for half an hour.
18. After half an hour, carefully remove it from the oven; now see clear lines.
    Note: Oil will likely also be present.

3.2.4. High Performance Thin Layer Chromatography (HPTLC)

HPTLC is a more advanced extension of TLC. HTPLC is based on TLC but has numerous enhancements, like finer particle sizes in the stationary phase, and it is at a more enhanced and advanced level of TLC. HPTLC is designed to enhance and improve the resolution of the compounds seen in TLC, along with higher sensitivity. The main difference between HPTLC and TLC lies in the sample application. As seen in section 3.2.3, a sample of phospholipids was spotted using a capillary. However, an HPTLC auto-sampler is used to spot the sample. HPTLC auto-sampler is a precision tool that applies a set amount of the sample by spraying it with nitrogen gas.

This method usually takes time as the HPTLC auto-sampler will spray small amounts. It is also important to mention that in HPTLC, the sample must be filtered with a syringe filter before being applied to the plate to ensure the syringe is not clogged or there is no remaining extract from previous trials.

3.2.5. Materials

Table 6. Materials for HPTLC

Materials:	Quantity:
Beaker	2
Chloroform	130 mL
Methanol	70 mL
Ammonia (25%)	2 mL
TLC Chamber	1
Saturation paper/Filter paper	1 sheet
Methanol	200 mL
Fridge	1
Copper Sulphate	20 g
Orthophosphoric Acid	8 mL
Sulfuric Acid	8 mL
Water Bath	1
TLC Paper	1 sheet
Cutter	1
Pencil	1
Ruler	1
Tweezer	1
Aluminium Foil	1 roll
Hair Dryer (optional)	1
Oven	1
HPTLC dipping chamber	1
Linomat 5 (any HPTLC machine)	1
Syringe (should be compatible with HPTLC machine)	1

3.2.6. Safety Considerations

- Ensure the need to wear goggles, gloves, a mask, and a lab coat throughout this experiment, and use correct PPE.
- Handle chloroform, methanol, ammonia, sulfuric and orthophosphoric acid in a fume hood and avoid inhalation as they are toxic if ingested.
- Exercise caution when using the cutter.
- Only use the machine with a lab assistant or someone aware of how to operate it.
- Handle the phospholipid extract with care when adding it to the syringe.

- If using a hair dryer on the plate, wait 5 minutes, as the heat from the fire can cause the vapours from the plate to ignite.

3.2.7. Method

1. In a beaker, 75 ml chloroform, 25 ml methanol, and 2 ml ammonia were prepared (25%). Mix well. This will act as the mobile phase.
2. Cut a piece of saturation/filter paper to the desired size of the TLC chamber and place it inside the chamber to saturate it.
3. Pour 10 mL of the mobile phase into both sides of the chamber, ensuring that the saturation paper is also run by the mobile phase.
4. Combine 200 mL of cooled methanol, 20 g of copper sulphate, and 8 mL of sulphuric acid to make the derivatisation reagent in a water bath to ensure the mixture does not go higher than 20°C.
5. Once the derivatisation reagent has been created, pour it into an HPTLC dipping chamber and cover it with aluminium foil.
6. Cut a rectangular piece out of TLC paper (12x5 cm) using a cutter so the lines are exact.
7. Place the HPTLC paper into the linomat 5, ensuring the dimensions are correct.
   Note: Use any HPTLC device; however, when conducting the trials, linomat 5 was used for HPTLC.
8. Take the syringe and rinse it with methanol and chloroform around 1-2 times.
9. Taking the phospholipid extract, draw and expel the extract using the syringe to ensure thorough cleaning.
   Note: It is crucial to ensure the phospholipid extract is compatible with the syringe.
10. Fill the syringe until around 83 mL (the remaining 3 ml are trial samples).
11. Place it on the machine and allow it to begin.
12. Once the extract has been spotted, tweezers are taken immediately and placed carefully into the TLC chamber.
13. Ensure that it is placed as linearly as possible.
14. Once placed, cover the chamber with foil and then the lid.
15. Keep an eye on the plate, and when 0.5 cm of the plate is yet to be run by the mobile phase, take a pair of tweezers and remove the plate from the chamber.
16. Allow the plate to dry until the mobile phase appears entirely gone.

    Note: To speed up the drying process, use a hair dryer.

17. Once the plate is dry, take a pair of tweezers and carefully dip the paper in the derivatisation reagent for 2 seconds.
18. Take it out and allow it to dry for 1 minute.
19. Put it in an oven and let it dry for half an hour.
20. After half an hour, carefully remove it from the oven; you should see clear lines.

    Note: Oil will likely also be present.

3.3. UV Spectrophotometer

A UV spectrophotometer measures the intensity of light absorbed across a variety of visible light and UV lights. A UV spectrophotometer can confirm phospholipids in coconut oil by first detecting and quantifying phosphorus in the coconut oil because phosphorus is a key component of phospholipids. The spectrophotometer emits a UV light that passes through the coconut oil sample, interacting with a blue-coloured complex that absorbs specific light wavelengths.

The remaining light that does not interact with the complex is detected, and the spectrophotometer calculates the absorbance, the difference between the emitted light and the detected light. The phosphorus concentration is found by comparing the sample's absorbance with the calibration curve; this concentration can then be linked to the quantity of phospholipids.

The methodology below converts phosphorus from the coconut oil's phospholipids into a form that the UV Spectrophotometer can detect and analyse (blue phosphomolybdic acid complex). The UV spectrophotometer method determines the phosphorus content by ashing in the presence of zinc oxide, and this is then followed by a spectrophotometer measurement of phosphorus as blue phosphomolybdic acid.

The methodology below relies largely on phosphorus being a large part of phospholipids, and the formation of the blue phosphomolybdic acid complex shows precise qualification of phosphorus and acts as a way to detect phospholipids from phosphorus. Thus, it provides a general overview of the phospholipid content in CNO.

Permission Statement for the Sections:
Under the Food Safety and Standards following Authority of India (FSSAI) copyright and reference policy --

"Material featured on this site may be reproduced free of charge in any format or media without requiring specific permission." The following Sections, 3.4.1, 3.4.2 and 3.4.3, have all been taken from the Revised FFSAI Manual of Methods of Analysis of Foods – reg (23.03.2021).

3.3.1. Materials

Table 1. Materials for UV Spectrophotometer

Materials:	Quantity:
Platinum basins/crucibles (should withstand temperature of 600°C)	1
Electric hot plate	1
Muffle Furnace	1
Watch glass (75 mm diameter)	1
Funnel Short Stemp (50 mm diameter)	1

Volumetric Flasks and glass stoppers (50 mL, 100 mL, 250 mL and 500 mL)	1 (per each quantity)
Pipette (10 mL)	9-10
Spectrometer with 1.0 cm cuvettes	1
Concentrated hydrochloric acid, sp. gr 1.18	5 mL
Zinc oxide, reagent grade	0.5 g
Potassium hydroxide, reagent grade	50 g
Concentrated sulphuric acid, sp. gr 1.18	140 mL
Sodium molybdate, reagent grade	12.5 g
Hydrazine sulphate (0.015%), reagent grade	0.150 g
Potassium dihydrogen phosphate, reagent grade, fried for 2 hr at 101°C	1.0967 g
Distilled water	2 L

3.3.2. Safety Considerations

- Potassium hydroxide: corrosive. Causes severe burns to the skin, eyes, respiratory tract, and gastrointestinal tract. Material is extremely destructive to all body tissues. It may be fatal if swallowed.
- Hydrochloric acid: Hazardous liquid which must be used with care. The acid itself is corrosive, and concentrated forms release acidic mists that are also dangerous. If the acid or mist comes into contact with the skin, eyes, or internal organs, the damage can be irreversible or even fatal in severe cases.
- Sulphuric acid: Concentrated sulphuric acid is extremely corrosive and can cause severe burns when not handled properly. This chemical is unique because it not only causes chemical burns but also secondary thermal burns as a result of dehydration. This dangerous chemical is capable of corroding skin, paper, metals, and even stone in some cases. If sulphuric acid makes direct contact with the eyes, it can cause permanent blindness. If ingested, this chemical may cause internal burns, irreversible organ damage, and possible death.
- Sodium molybdate: It may cause eye, skin and respiratory tract irritation. It may be harmful if swallowed, inhaled or absorbed through the skin.

3.3.3. Method

Preparation of Reagents:

1. Sodium molybdate – Carefully add 140 mL of concentrated sulphuric acid to 300 mL of distilled water. Cool to room temperature and add 12.5 g of Sodium molybdate. Dilute to 500 mL with distilled water. Mix thoroughly and allow to stand for 24 hours before use.
2. Hydrazine sulphate – 0.015%. Dissolve 0.150 g hydrazine sulphate in 1 L water.
3. Potassium hydroxide – 50% solution. Dissolve 50 g KOH in 50 mL distilled water.
4. Standard phosphate solution:
   a. Stock solution – Dissolve 1.0967 g of dry Potassium dihydrogen phosphate in distilled water and make 250 mL in a volumetric flask. The solution contains 1 mg phosphorous per mL.
   b. Working Solution – Dilute 5 mL of standard stock solution with distilled water to 500 mL in a volumetric flask. This solution contains 0.01 mg phosphorus per mL.

Method of analysis:

1. Weigh 3-4 g of sample accurately in a crucible or Pt basin.
2. Add 0.5 g Zinc oxide and heat slowly on the hot plate until the sample thickens, then gradually increase the heat until the mass is completely charred.
3. Place in a muffle furnace at 550 – 600 degrees Celsius and hold for 2 h.
4. Remove and cool to room temperature.
5. Add 5 mL distilled water and 5 mL hydrochloric acid to the ash.
6. Cover the crucible with a watch glass and heat gently to boiling for 5 min.
7. Filter the solution in a 100 mL volumetric flask. Wash the inside of the watch glass and the crucible with about 5 mL of hot water using a wash bottle with a fine steam of water. Wash the crucible and filter paper with 4 additional portions of hot distilled water.
8. Cool the solution to room temperature and neutralise to a faint turbidity by adding 50% KOH solution dropwise.
9. Add concentrated hydrochloric acid dropwise until the precipitate is just dissolved, then add 2 additional drops.
10. Dilute to volume with water and mix thoroughly.
11. Pipette 10 mL of this solution into a clean, dry 50 mL volumetric flask.
12. Add 8 mL of hydrazine sulphate solution and 2 mL of sodium molybdate solution in this order.
13. Stopper and invert 3-4 times.
14. Loosen the stopper and heat for 10 ±0.5 min in a vigorously boiling water bath.
15. Remove from bath, cool to 25 ±5 °C in a water bath, dilute to volume and mix thoroughly.
16. Transfer the solution to a clean, dry cuvette and measure the absorbance at 650 nm in a spectrophotometer adjusted to read 0% absorbance (100% transmittance) for distilled water.
17. Prepare a reagent blank without the oil test sample. Measure the phosphorus content of the sample and the blank compared with the standard curve.
18. Prepare the standard curve-pipette 0.0, 1.0, 2.0, 4.0, 6.0, 8.0 and 10.0 mL of standard working solution into the 50 mL volumetric flasks. Dilute each to 10 mL with distilled water using a measuring pipette.
19. Add hydrazine sulphate and sodium molybdate as above. Plot the absorbance of each standard against its phosphorus content in mg on a linear graph paper, observe the relationship, and confirm the presence of phospholipids.

4. Results and Discussion

4.1. Thin Layer Chromatography (TLC)

The TLC method required extensive trial and error by referring to past studies and experiments. Creating a mobile phase that could efficiently carry the components of the coconut oil with it was necessary to see the presence and quantity of phospholipids present.

However, once that had been completed, phospholipids appeared, and it was also possible to see the different phospholipids present, as they each had different affinities. While TLC is a much simpler method, it quantifies and confirms phospholipids, even going so far as to distinguish which kinds of phospholipids are present lightly. When conducting preliminary rounds of research before beginning the actual experiment, a study named *"Separation of Phospholipids by HPTLC – An Investigation of Important Parameters"* [4] published in 2008, provided a standardised mobile phase to use for the separation of phospholipids, which can also be used in this experiment, to confirm the presence of phospholipids.

The study quoted: "*Separation of the phospholipids PA, PC, PE, PI, LPA, LPC, LPE, and LPI can be achieved on HPTLC silica gel 60 (Merck) with chloroform, methanol, water, ammonia 25% (60:34:4:2) as mobile phase."* The first time the experiment was conducted, HPTLC silica gel 60 was used to prepare the mobile phase accurately. However, through numerous trials of TLC, the phospholipids did not appear correctly on the plates, and instead, large amounts of oil were travelling up with the solvent front. In Figure 8, the coconut oil can be seen moving up the solvent front and combining it with the phospholipids, which does not allow for proper results or evaluation. However, in Figure 9, the phospholipids can be seen only with minimal oil, allowing for concluding proper results.

In Figure 9, a clear distinction between the phospholipids can be observed, which cannot properly be seen in Figure 8. This was achieved through altering the quantity of solutions in the mobile phase. It was done by increasing the amount of chloroform, as chloroform is a non-polar solvent and by increasing the quantity, the mobile phase became more non-polar. As coconut oil is largely non-polar (due to triglycerides), in a more non-polar mobile phase, the coconut oil would be unable to travel well and will mostly only stay at the starting point. Also, reducing the amount of methanol (a polar solvent) made the mobile phase even more non-polar and thus reduced the solubility of the non-polar triglycerides and, therefore, reduced their overall mobility, not allowing the oil to travel up effectively.

Removing the water was done as ammonia (25%) contains amounts of water. Once changing the quantities had been completed numerous times, this was the most effective mobile phase for showing the phospholipids clearer and not showing oil.

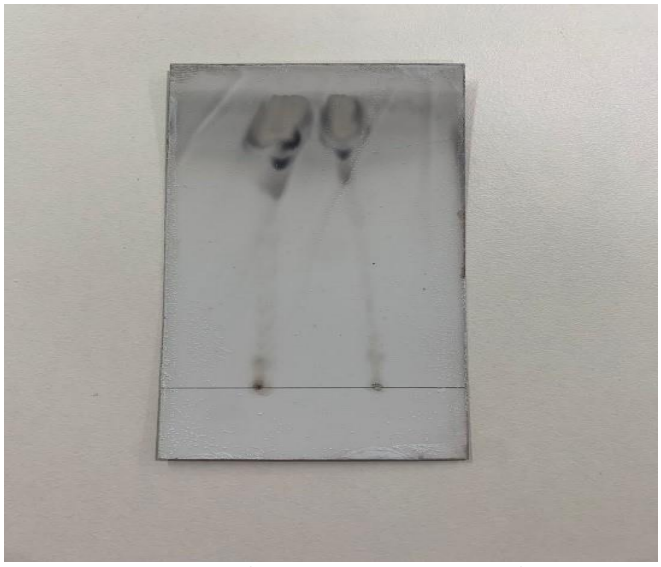


Fig. 8 TLC with first mobile phase (less chloroform)

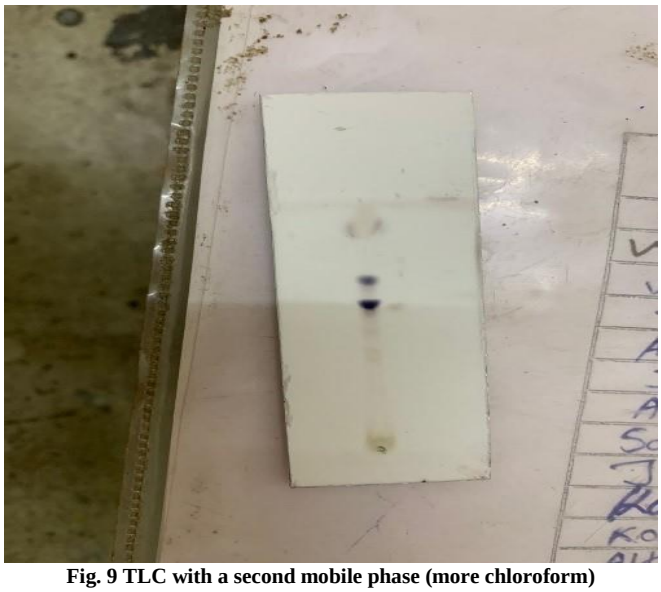


Fig. 9 TLC with a second mobile phase (more chloroform)

4.2. High Performance Thin Layer Chromatography (HPTLC)

Due to the similarity between TLC and HPTLC, the methodology and trial and error did not vary too much, as they are both based on the same principle. Just one is more precise. Due to the greater precision this method required, it proved challenging to understand how much oil to spot and eliminate air bubbles from the syringe used to apply the extract. HPTLC proved more accurate than TLC, as phospholipids were seen much clearer, and the distinction between the phospholipids was also clearer. HPTLC is a timely process requiring precision as it relies heavily on the machine but provides clear results.

Note: The same challenges are faced in TLC 4.1. was also present for this experiment. As seen in Figure 11, the more

chloroform present in the mobile phase, the greater the amount of oil moved up the solvent front along with the phospholipids. While in HPTLC, the phospholipids are initially much more present, especially towards the baseline, as it moves up, the oil and phospholipids combined, not allowing for proper evaluation. As seen in Figure 11 (the mobile phase is not completely dry), there is a clear distinction between the phospholipids and very minimal amounts of oil compared to Figure 10 because the mobile phase in Figure 11 was different and contained more chloroform. As done in TLC to get a starting point of the experiment, in HPTLC, the *"Separation of Phospholipids by HPTLC – An Investigation of Important Parameters"*, published in 2008, was referred to. It provided the quantity of extract sprayed on by the phospholipid extract— "*The general SOP for HPTLC as previously published was followed: Sample volumes of 2-10 mL were applied as 8mm bands using the spray-on technique."*

Fig. 10 HPTLC with first mobile phase (less chloroform)

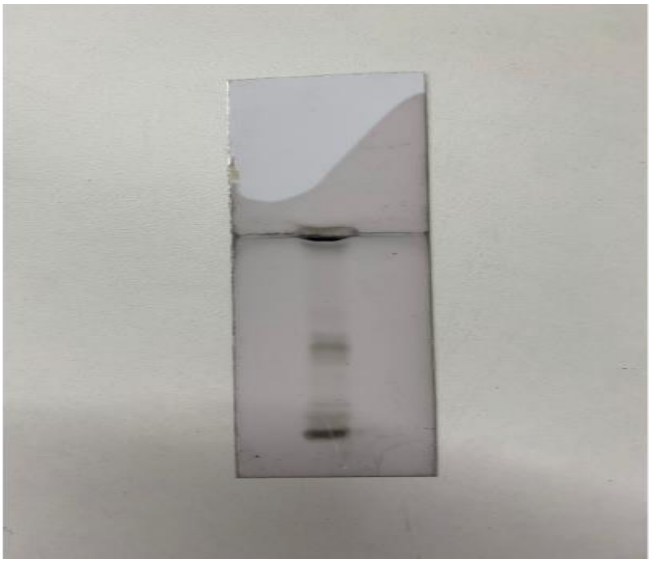


Fig. 11 HPTLC with second mobile phase (more chloroform)

The same settings had been followed initially; however, it was found that only using 10 mL of the phospholipid extract was not enough to get proper results. As seen in Figure 12 (b), the HPTLC on the right has a much darker and more transparent coconut oil and phospholipid distinction than Figure 12 (a) on the left. Figure 12 (a) has 50 mL of phospholipid extract, whereas Figure 12 (b) has 80 mL of phospholipid extract. Through trial and error, it was understood that for the extract 70-80 mL of extract was suited for best results rather than mentioned in the study.

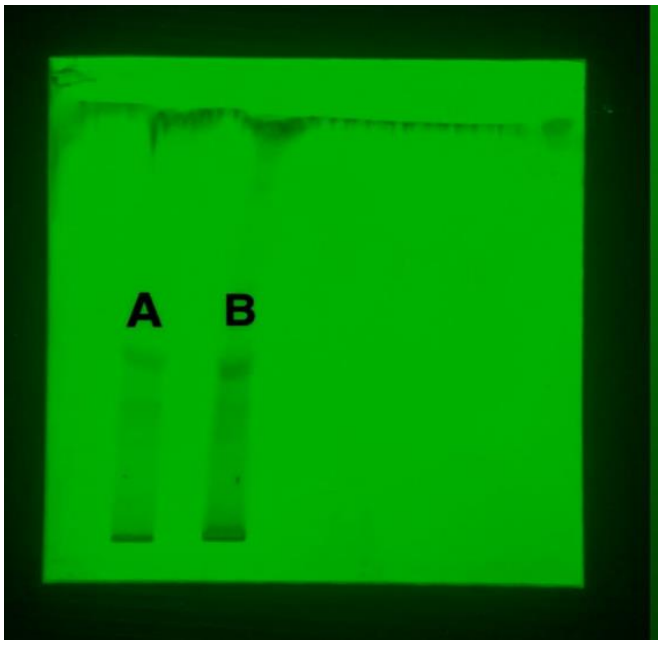


Fig. 12 HPTLC is seen under UV light. (a) Less concentration, (b) More concentration

Increasing the amount of extract also led to the syringe needing to hold more. This increase in quantity caused the extract in the syringe to develop air bubbles. These were extremely challenging to remove, as pushing out the extract was the only way to get them out. In one of the first trials of HPTLC, due to an unnoticed air bubble, the machine stopped, and the trial had to be done from the beginning. That was challenging. However, through multiple trials, the air bubbles were efficiently extracted. These results and methodology can be compared to the study by Touchstone et al. (1980). While the study is older, it can be used as a baseline point for TLC in more modern applications. The Touchstone study aims to refine the separation process and has proved essential for critical applications in food, science, nutrition, and lipid profiling. In the Touchstone study, their final method combines solvents to achieve the most distinct separation of phospholipid components. It compares to the TLC data, as both studies show rigorous processes in finding the best mobile phase. The Touchstone research focuses on more pre-established solvent combinations; however, the TLC method in this study focuses on a trial-and-error method. In both studies, TLC proved to separate the phospholipids effectively;

however, as the Touchstone study had a more refined system and less trial-and-error, they had less interference. This study showed that combining multiple is more efficient instead of finding simply one mobile phase that works. It also presents a potential hypothesis on whether each medium that the phospholipids are in needs a different mobile phase.

4.3. UV Spectrophotometer

The UV Spectrophotometer varied the most from the two methods, as it was the only one that did not require the principle of chromatography. Due to this method's complex nature, it was impossible to perform the experiment in the lab, so the experiment was conducted in another lab that specialised in this specific methodology. UV Spectrophotometer was the only method that provided quantitative data, which proved to be more accurate than the other two, as actual numbers were used to quantify and confirm the presence of phospholipids. This would prove the most useful in this research paper, as this method would provide accurate values on what quantity of phospholipids can be removed while ensuring the oil is not stripped of nutrients. The formation of the blue phosphomolybdic acid complex relies largely on the ashing/charring of zinc oxide. The zinc oxide is heated between temperatures 550°C - 600°C (extremely high temperatures). This high temperature requires specific equipment but is a safety hazard for others in the laboratory. Due to this, the experiment could not be performed in the current lab, and it had to be assisted at another lab. As it was impossible to get hands-on experience and conduct the experiment multiple times, it did not allow for proper analysis and thorough understanding as the others. Nevertheless, being assisted by someone who was an expert at conducting this specific experiment meant that it was the least time-consuming.

Note: Challenges were kept to a minimum due to assistance from someone who was an expert at this specific experiment.

Table 8. Raw data from UV Spectrophotometer

Standard	Concentration	Absorbance
1	0	0.01
2	0.2	0.059
3	0.4	0.11
4	0.8	0.233
5	1.2	0.358
6	1.6	0.487
7	2	0.61

Table 4 quantifies the presence of phospholipids. As colour intensity is directly proportional to the concentration of phospholipid quantity in coconut oil, and greater concentration means greater absorbance of phospholipids, it can thus be verified that as the concentration of the phospholipid in the standard is increased, the absorbance increases, stating a clear, linear relationship. Figure 13 (from left to right) demonstrates the increase in colour intensity. Start from the left with standard one and on the right with standard 7. The increase in colour intensity is a qualitative observation that can be used to quantify and verify the presence of phospholipids.

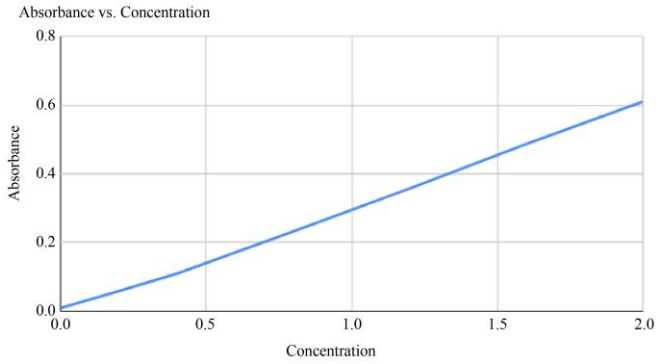


Fig. 13 Absorbance vs concentration plotted

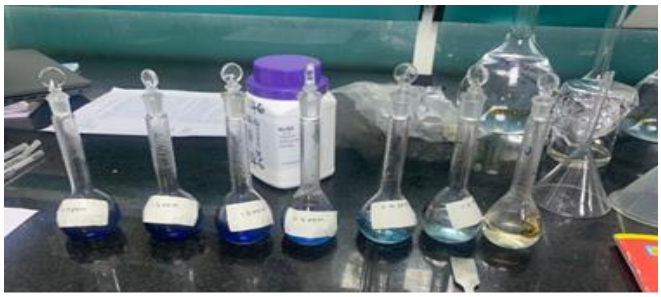


Fig. 14 Decreasing color intensity leads to a lower concentration of phospholipids

The UV spectrophotometer method can be compared to "*Determination of Phospholipids in Vegetable Oil by Fourier Transform Infrared Spectroscopy"*, published in the Journal of the American Oil Chemists' Society. Fourier Transform Infrared Spectroscopy (FTIR) is similar to UV spectrophotometry as they both are advanced and more precise techniques used to identify and quantify various components. FTIR measures the absorbance of infrared radiation by individual chemical bonds in a molecule. This relies on the principle that different functional groups have distinct spectral signatures and absorb at different and specific wavelengths, allowing for more precise quantification of types of phospholipids and quantity, as it can detect even the smallest amount. As seen in the study "*Determination of Phospholipids in Vegetable Oil by Fourier Transform Infrared Spectroscopy"* study, FTIR is significantly more complex than UV spectrophotometer, along with being extremely costly and requiring the analysis of spectra to be done by professionals in the field. In comparison, a UV spectrophotometer is more straightforward and accessible in labs. Comparing the two results, the study done on FTIR also showed a positive correlation between concentration and absorption, as seen in Figure 13**.** However, FTIR also measured the molecular vibrations that appeared as absorption peaks at specific wavelengths, as distinct peaks correspond to

functional groups. E.g. carbonyl, phosphate and hydrocarbon chains. The study also took the analysis a step further and saw the differences in phospholipid compositions based on refining and heating and nothing related to vegetable oil. This helped to understand which specific peaks may increase or shift, indicating how the quantity and measurements of phospholipids can also shift based on what is done to the oil. It would have allowed for a deeper understanding of the phospholipids in coconut oil, as this study does not see how refined, processed and natural coconut oil vary.This could be a step further taken to understand which phospholipids are necessary and which are not.

5. Conclusion

5.1. Evaluation of Each Method of Analysis

TLC was the easiest method to set up and execute, with the most minor equipment required, meaning that most people can do it with a lab. It was much more cost-effective than the other methods and provided a visual confirmation similar to that of HPTLC. However, when comparing the TLC plates to the HPTLC plates, it is evident that TLC showed lesser sensitivity. In TLC, it was harder to see the smaller traces of phospholipids present in HPTLC. Rather than precise quantification data, it provided qualitative data, the latter being preferred. The preparation and setup of TLC are largely done by hand, thus making it more prone to human error. However, TLC proved extremely useful in confirming the process of phospholipids in the extract. When doing trial and error to understand the most efficient method of extracting phospholipids from coconut oil (centrifuging, hexane, orthophosphoric acid, etc.), TLC proved the fastest method to confirm whether the extraction was efficient. Similarly, this was seen with the mobile phase. Once it had been finalised how to extract the phospholipids, finding the best mobile phase was key, and testing it with TLC proved to be the fastest way to understand. HPTLC in this experiment largely acted as an extension and more complex TLC.

As multiple trials and errors had been undertaken regarding phospholipid extraction and composition of the mobile phase, executing HPTLC was mostly simple. Any issues like excess coconut oil travelling up the solvent front were resolved while doing TLC (as it was the faster method); however, for each extract and mobile phase, it was also ensured to test it with HPTLC to see if there were any differences (which was not found). HPTLC proved to show a better composition and breakdown of the phospholipids than TLC. HPTLC has very high sensitivity and resolution due to the specialised equipment (linomat 5), which leads to precise application and clearer visualisation and confirmation of the phospholipids. As a machine did the spotting, it removed human errors, leading to a fairer experiment. However, it was found that the 80 □L was spotted on the plate because the syringe is extremely precise with an application; it took a significant number of times. Multiple samples had to be spotted and tested, making HPTLC more time-consuming.

However, HPTLC combines high sensitivity and accuracy and reduces human errors, leading to one of the most fair and accurate analysis methods. As the UV Spectrophotometer method includes ashing, conducting this within the current laboratory was prohibited. While this did not stop the investigation, it just meant that conducting as many trials as preferred was impossible. Upon observation, the UV Spectrophotometer is one of the simplest and most straightforward analysis methods, as it quantifies and demonstrates relationships in phospholipids using high-technology equipment and relationships. UV spectrophotometer is also not as time consuming as HPTLC, as this method only verifies a relationship and quantifies phospholipids.

It is much quicker to perform. Factoring in that this is an FSSAI method already pre-given, it also does not involve any of the trial and error that TLC and HPTLC did, making it easier and faster to conduct and conclude. However, while this is an excellent quantification method representing the expected linear relationship, phospholipids are much more complex particles than just part of phosphorus, which the UV spectrophotometer generalises. Furthermore, this process is extremely labour and technique intensive, which requires a trained professional to conduct the experiment, especially with things such as filtering, weighing, neutralising, etc. The equipment must be frequently calibrated throughout the experiment after each test, as the UV spectrophotometer is incredibly sensitive and can catch anything. For anyone who is simply attempting this experiment, it is, therefore, likely they will face interference from not calibrating correctly.

5.2. Result Enhancements and Benchmarking Against Literature

While there is already literature surrounding the separation of phospholipids, this study proved unique and helpful as opposed to already existing literature and state-of-the-art techniques due to the customization of the mobile phase, multi-technique approach, use of both quantitative and qualitative methods of analysis to be able to weigh the pros and cons and by directly addressing specific challenges posed by coconut oil, the study went beyond the uses of older techniques, providing a clearer, more reproducible and reliable results. One of the biggest breakthroughs from this study was modifying the mobile phase. This modification was tailored for a unique composition of coconut oil, abundant in triglycerides. In earlier literature- the 2008 study published in the Journal of Liquid Chromatography & Related Technologies called: *"Separation of Phospholipids by HPTLC – An Investigation of Important Parameters"* the study identified a standard mobile phase (chloroform, methanol, water and ammonia). However, this study noted the high triglyceride content in the coconut oil, which required a more non-polar mobile phase to minimize interference. Increasing the amount of chloroform and reducing methanol, this study minimised the movement of non-polar triglycerides,

leading to a clearer and more noticeable separation of phospholipids.

Furthermore, the 2008 study focused solely on separating phospholipids by HPTLC, not recognizing a cheaper and faster alternative following the same principle. This study achieved the best mobile phase by incorporating a more cost-effective method to test out different and new mobile phases, as TLC had done. This study also recognized that there is no standard mobile phase. Thus, employing TLC allowed anyone hoping to pursue a similar study to find their mobile phase as fast as possible efficiently. Lastly, this study employed a qualitative approach, opposing the two analysis methods observed in this paper.

A UV spectrophotometer allowed the study to feature precise numerical data and observable relationships. By referring to the *Revised FFSAI Manual of Methods of Analysis of Foods – reg (23.03.2021) section 3.4.1, 3.4.2 and 3.4.3*, this study was able to adapt a determination of phosphorus in soya bean oil to fit coconut oil. The methodology from the FSSAI did employ a UV spectrophotometer. It gave this study the idea of using the link between the formation of a blue phosphomolybdic acid complex to detect phosphorus, as phosphorus is a significant component in phospholipids. This study took it further, observing the qualitative relationship (numerical values) and the quantitative values (the increasing colour). Recognising and applying this link to coconut oil instead of soya bean oil pushed this study forward and showed a fair comparison between all analysis methods.

Acknowledgments

Audentes and Analytica's Pvt Ltd for helping execute the UV Spectrophotometer method.

References

[1] Abdelmoneim H. Ali et al., "Natural Phospholipids: Occurrence, Biosynthesis, Separation, Identification, And Beneficial Health Aspects," *Critical Reviews in Food Science and Nutrition*, vol. 59, no. 2, pp. 253-275, 2017. [CrossRef] [Google Scholar] [Publisher Link]

[2] Marta I. V. Brevedan, Amalia A. Carelli, and Guillermo H. Crapiste, "Changes in Composition and Quality of Sunflower Oils During Extraction and Degumming," *Grasas Aceites*, vol. 51, no. 6, pp. 417-423, 2000. [Google Scholar] [Publisher Link]

[3] Amalia A. Carelli, Marta I. V. Brevedan, and Guillermo H. Crapiste, "Quantitative Determination of Phospholipids in Sunflower Oil," *Journal of the American Oil Chemists' Society*, vol. 74, pp. 511-514, 1997. [CrossRef] [Google Scholar] [Publisher Link]

[4] D. Handloser, V. Widmer, and E. Reich, "Separation of Phospholipids by HPTLC - An Investigation of Important Parameters," *Journal of Liquid Chromatography & Related Technologies*, vol. 31, no. 13, pp. 1857-1870, 2008. [CrossRef] [Google Scholar] [Publisher Link]

[5] Dwi Hudiyanti et al., *Coconut Phospholipid Species: Isolation, Characterization and Application as Drug Delivery System*, Nano- and Microencapsulation - Techniques and Applications, 2019. [CrossRef] [Google Scholar] [Publisher Link]

[6] Dwi Hudiyanti1, Muhammad Fuad Al Khafiz, and Khairul Anam, "Coconut (Cocos nucifera L.) Lipids: Extraction and Characterization," *Oriental Journal of Chemistry*, vol. 34, no. 2, pp. 1136-1140, 2018. [CrossRef] [Google Scholar] [Publisher Link]

[7] Jing Li et al., "A Review on Phospholipids and Their Main Applications in Drug Delivery Systems," *Asian Journal of Pharmaceutical Sciences*, vol. 10, no. 2, pp. 81-98, 2015. [CrossRef] [Google Scholar] [Publisher Link]

[8] Revised FSSAI Manual of Methods of Analysis of Foods, Food Safety Standards Authority of India, 2021. [Online]. Available: chrome-extension://efaidnbmnnnibpcajpcglclefindmkaj/https://fssai.gov.in/upload/uploadfiles/files/Manual_Revised_Oil_Fats_22_06_2021.pdf.

[9] Nwankwojike Bethrand Nduka, O. Onuba, and Uchechukwu Omah Ogbonna, "Development of a Coconut Dehusking Machine for Rural Small Scale Farm Holders, *International Journal of Innovative Technology and Creative Engineering*, vol. 2, no. 3, pp. 1-7, 2012. [Google Scholar] [Publisher Link]

[10] Cyberlipid, Quantitative Determination of Phospholipids, 2014. [Online]. Available: https://cyberlipid.gerli.com/techniques-of-analysis/analysis-of-complex%20lipids/phospholipid-analysis/quantitative-estimation-of-pl/#:~:text=A%20quantitative%20determination%20of%20the,must%20be%20multiplied%20by%201

[11] Lukas Schwingshackl, and Georg Hoffmann, "Monounsaturated Fatty Acids and Risk of Cardiovascular Disease: Synopsis of The Evidence Available from Systematic Reviews and Meta-Analyses," *Nutrients*, vol. 4, no. 12, pp. 1989-2007, 2012. [CrossRef] [Google Scholar] [Publisher Link]

[12] C. Paquot, and A. Hautfenne, *Standard Methods for the Analysis of Oils, Fats and Derivatives*, International Union of Pure and Applied Chemistry, Blackwell Scientific Publications, Oxford, 7th ed., 1992. [Google Scholar] [Publisher Link]

[13] Tong Wang, Earl G. Hammond, and Walter R. Fehr, "Phospholipid Fatty Acid Composition and Stereospecific Distribution of Soybeans with a Wide Range of Fatty Acid Composition," *The Journal of the American Oil Chemists' Society*, vol. 74, pp. 1587-1594, 1997. [CrossRef] [Google Scholar] [Publisher Link]

[14] Jun-ichiro Yoshino et al., "Chemical Structure of a Novel Aminophospholipid from Hydrogenobacter thermophilus Strain TK-6," *Journal of Bacteriology*, vol. 183, no. 21, pp. 6302-6304, 2001. [CrossRef] [Google Scholar] [Publisher Link]