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Phytochemical Analysis of Green Leaves Through Column Chromatography

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Abstract: ***Chromatography has been a fundamental technique used for chemical separation that dates back to the 1830s. Specifically, column chromatography, typically taught in introductory organic chemistry laboratories, traditionally involves the use of halogenated or harmfull solvents, which novice students often over use. This situation runs contrary to the principles of responsible chemical and waste management emphasized by the green chemistry movement. Since this movement began, conventional means of separation using harmful solvents have been modified to emphasize the need for safer, less hazardous materials and the generation of such waste. The current experiment emphasizes the green chemical principles of renewable feed stocks and recycling to minimize waste, while simultaneously introducing or reinforcing common organic techniques, including solvent extraction, column chromatography, and thin - layer chromatography for the isolation and identification of photosynthetic pigments from spinach leaves. Students gain practical experience processing plant material to isolate and identify the pigments, β - carotene, xanthophylls, and chlorophylla, using the solvents hexane and acetone.***

Keywords: Column Chromatography, Green Chemistry, Hazardous materials, Thin Layer Chromatography, Photosynthetic Pigments**,** β - Carotene, Xanthophylls, and Chlorophylla

1. Introduction

Chromatography is the science which studies the separation of molecules based on differences in their structure and composition.1Column chromatography is a technique which is used to separate a single chemical compound from a mixture dissolved in a fluid. It separates substances based on differential adsorption of compounds to the adsorbent as the compounds move through the column at different rates which allow them to get separated in fractions. This method is a type of adsorption chromatography technique.2When the mobile phase along with the mixture that needs to be separated is introduced from the top of the column, the movement of the individual components of the mixture is at different rates. The components with lower adsorption and affinity to stationary phase travel faster when compared to the greater adsorption and affinity with the stationary phase. The components that move fast are removed first whereas the components that move slowly are eluted out last. Depending of the stationary phase the Column Chromatography divided into following types: Adsorption Column Chromatography, Partition Column Chromatography, Ion exchange Column Chromatography. In Adsorption Column Chromatography the adsorbent packed in the column acts as a stationary phase, whereas in Partition Column Chromatography the liquid supported by the adsorbent act as stationary phase. In Ion exchange Column Chromatography, the column is packed with ion exchangers. The major advantages of column chromatography are its ability to handle larger amounts of material (compared to GC, TLC and HPLC) and the ability to change the eluting solvent throughout the course of the separation. This allows one to remove one component while a desired product remains essentially unmoved. A change in the mobile phase moves the desired product through the column, which may include changes in solvent polarity, changes in pH, or changes in ionic strength. The last two are used largely in biological separations (proteins, peptides). The stationary phase serves as an adsorbent through which the mobile phase is passed. Many compounds with varying functional groups may be used as the stationary phase and several types of interactions can aid in developing the desired separation (i. e., hydrogen bonding, dipole interactions, electrostatic interactions, Van der Waals forces, size exclusion, affinity, etc.). The following sequence illustrates the general affinity of the functional groups towards a polar stationary phase like silica. A Column may be packed by (i) Dry packing / dry filling, in this the required quantity of adsorbent is poured as fine dry powder in the column and the solvent is allowed to flow through the column till equilibrium is reached. (ii) Wet packing / wet filling, in this, the slurry of adsorbent with the mobile phase is prepared and is poured into the column. It is considered as the ideal technique for packing.

Adsorption Column Chromatography is a green approach to analyse the phytopigments of green leaves. This green chemical principle involves renewable feedstock and recycling to minimize waste.[3, 4] Green leaves may be taken as Spinach, which is a renewable feedstock that serves as a raw material for isolation, separation and identification of phytopigments. The residual waste of Green leaves or Spinach are a non - hazardous solid that offers inexpensive disposal and safe handling. Green leaves or Spinach leaves are affordable and the pigments are readily extractable using acetone, a common cost effective non - halogenated organic solvent.5The green leaves or Spinach leaves are treasures of phytochemicals. Phytochemicals are chemicals of plant origin. Phytochemicals (from Greek *Phyto*, meaning "plant") are chemicals produced by plants.4Phytochemicals are colourful parts. They are involved in photosynthesis. They are classified as Chlorophyll, Carotenoid and Xanthophyll. Chlorophylls, the green pigments, absorb certain wavelengths of light that are then converted into chemical energy. Carotenoids, the yellow pigments found in spinach, are also involved in photosynthesis. Carotenoids are part of a

larger collection of plant derived compounds called terpenes. Carotenoids are tetraterpenes.6, [7]Xanthophylls are oxygen - containing derivatives of the carotenes. The Spinach leaves contain Chlorophyll*a*, Chlorophyll*b* and *β* Carotenoid as major pigments as well as other smaller pigments as Xanthophyll.8 Column chromatography is one of the most useful methods for the separation and purification of both solids and liquids.[9, 10]Its major application includes, separation of mixture of compounds, removal of impurities or purification process, isolation of active constituents, isolation of metabolites from biological fluids, estimation of drugs in formulation or crude extracts.[11, 12]The three spinach pigments isolated by column chromatography and identified byTLCusinga60: 40hexanes/acetone solvent system in the developing chamber.1[3, 14]

2. Material and Method

(i) Instrumentations: Column Chromatography consist of

- **A Column**: A column is a 20 - 100 cm long and 1 - 4cm internal diameter and made of glass tube drawn out at one end that is provided with a stopcock to regulate the flow of eluent through the clamp. They may be either of the conventional type filled with the stationary phase, or of the microbore type in which the stationary phase is coated directly on the inside wall of the column. After packing, a paper disc kept on the top, so that the stationary layer is not disturbed during the introduction of sample or mobile phase.
- **A stationary phase**: Chosen to be appropriate for the analytes to be separated.
- **A mobile phase and delivery system**: Chosen to complement the stationary phase and hence to discriminate between the sample analytes and to deliver a constant rate of flow into the column.
- **An injector system:** To deliver test samples to the top of the column in a reproducible manner.
- **A detector:** To give a continuous record of the presence of the analytes in the eluate as it emerges from the column.
- **A fraction collector:** For collecting the separated analytes for further biochemical studies.
- Beaker
- Funnel
- Erlenmeyer flask 50 mL
- Pipette 10 mL
- Mortar and pestle
- Clamps, Stand

(ii) Chemical Required:

- Chromatography Tube (about 25 cm X 1, 7 cm diameter)
- Calcium carbonate or Silica Gel (as Packing Material)
- Extract of Green Leaves or Spinach Leaves
- Hexane and Acetone (as Mobile Phase)
- Petroleum Ether (to prepare crude or extract of Green Leaves or Spinach Leaves)

(iii) Method

Method consists of following steps:

Packing of Column

(i) Took a cylindrical glass column and plugged in a small piece of cotton
(ii) Thoroughly cleaned the glass tube/column with Chromic acid, rinsed it with tap water and then with distilled water.
(iii) Mounted the column with stand.
(iv) With the lower end closed, poured some solvent into the tube so that it is about one third full.
(v) The column was first filled with a non polar solvent.
(vi) Prepared a thin slurry of the packing material (25g of fresh Silica Gel in 100 ml of Hexane).
(vii) Poured the slurry into the column.
(viii) Placed a conical flask below the mounted column and drain out the excess solvent (hexane).
(ix) Closed the stop cock when the level of the solvent reaches just above the settled Silica Gel.

Preparation of the crude extract:

(i) 10 grams of dry spinach leaves were weighed. (All stems and veins were removed from the leaves before weighing).
(ii) The spinach leaves were then crushed and ground using a mortar and pestle and made into a fine paste by adding 10mL (50: 50) mixture of hexane with acetone.
(iii) The filtrate from the well crushed paste transferred into a conical flask through a funnel that is plugged with a small piece of cotton.
(iv) Added 2 - 3 spatula of anhydrous sodium sulphate to remove traces of water present in the crude extract.
(v) Filtered off sodium sulphate from the crude extract using a funnel.

Adsorbing the Crude filtrate to Silica gel:

(i) Added about 3g of silica gel (for column chromatography 60 - 120 mesh) to the crude extract.
(ii) Gently heated the mixture using a hair dryer until the silica gel became free flowing.

Loading the crude extract on to the Column:

(i) Transferred the adsorbed crude material into the solvent layer above the silica gel in the packed column.

Development of the Column:

(i) After the application of crude material to the column, column was then irrigated with alcohol, coloured bands were observed in descending order of their absorptivity.
(ii) Two green zone followed by three yellow rings were appeared at the top of the column.
(iii) These bands were in descending order.
(iv) These were due to Chlorophyll B (Pale Yellow), Chlorophyll A (Green Blue), Xanthophyll (Yellow) and β - Carotene (Yellow) respectively.

Elution from the Column:

(i) **Elution using Hexane**: Continue filled the column with hexane and eluted until the yellow coloured β - Carotene ran down the column. As the elution progresses, β - carotene eluted out of the column, collected this in a conical flask.
(ii) **Elution using Hexane and Acetone**: Continue filled the column with mixture of hexane and Acetone and eluted until the yellow - coloured Xanthophyll ran down

the column. As the elution progresses, Xanthophyll eluted out of the column, collected this in a conical flask.

(iii) **Elution using Acetone:** After collecting the yellow pigment, filled the column with acetone. Placed another conical flask below the mounted column. As elution with acetone progresses, the green pigments started moving down the column. Collected Green pigment as Chlorophyll in the conical flask.

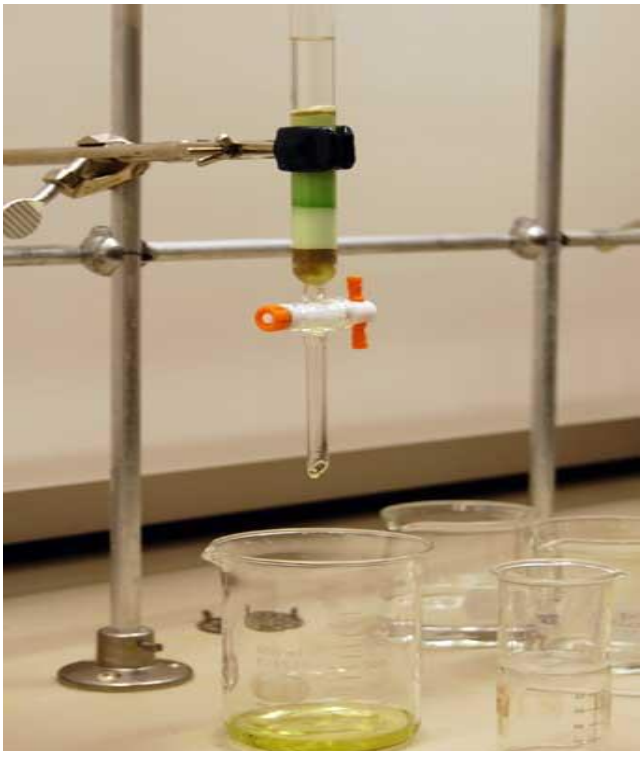
Removal of Solvent:

(i) The yellow and green pigments collected from the column were then concentrated by removing the solvents using a rotary evaporator.

(ii) The pigments left behind in the round bottomed flask after rotary evaporation are transferred into watch glasses using spatula.

3. Result and Discussion

The extraction and identification of pigments from spinach experiment was completed within 2 h. The pigments extracted from spinach provided a visual means to monitor separation and elution by column chromatography. Using a mobile phase of 100% hexane, the first band to pass through the column was β - carotene, which had a yellow to orange color. Once the β - carotene band had been collected, the eluting solvent was changed to 90: 10 hexane/acetone (v/v) to elute the pigment consisting of a yellow band of xanthophylls. Upon collection of the second band, the eluting solvent was changed to acetone to elute the remaining polar pigments consisting of a first band for blue - green chlorophyll *a*and a second band of bright green chlorophyll *b*. Chlorophyll *b* was typically not collected for analysis. The yellow and green pigments were isolated from the column. Green pigments are chlorophylls and yellow pigments are carotenoids and Xanthophyll.



4. Conclusion

Column Chromatography is a conventional tool for separation of phytochemicals, removal of impurities. This experiment was used solvent extraction, column chromatography to extract, isolate, andidentify plant pigments. Effective separation of constituents from different sources in preparative scale can be achieved by Column Chromatography. Availability of wide range of stationary phases makes the technique to be used for different kind of mechanisms. It is simultaneously introducing the green principles of renewable feed stocks and recycling to minimize waste. Understanding the basic principle of Column Chromatography enables us to find solutions for current research problems.

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Conflicts: There is no conflict

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