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Applications of Column, Paper, Thin Layer and Ion Exchange Chromatography in Purifying Samples: Mini Review

Enyoh CE[1,2], Isiuku BO[2], Verla AW[1,2]*

[1]Group Research in Analytical Chemistry, Environment and Climate Change (GRACE&CC), Department of Chemistry, Imo State University (IMSU), Imo State, Nigeria

[2]Department of Chemistry, Imo State University (IMSU), Imo State, Nigeria

Abstract

An important technique which allows purification of mixture components is chromatography based on interaction between a stationary and mobile phase. The mixture components redistribute themselves between the phases either adsorption, partition, ion exchange or size exclusion. Here, we presented a review of applications of column, paper, thin layer and ion exchange chromatography in purifying samples: The technique has wide use in the analysis of proteins molecules, nucleic acids, drugs, antibiotics and biological matrices and does not require the use of machines or special devices, it is fully portable and easy to handle and considerably cheaper than most commercial methods.

Keywords: Chromatography; Column chromatography; Protein purification; Purification; Separation; Drugs

Background: Historical Perspective

It was an Italian-born scientist by the name Mikhail Tsvet, who first used chromatography in 1900 in Russia [1], to primarily separate pigments present in plant (chlorophyll, carotenes, and xanthophylls). Mikhail Tsvet continued to use the method throughout the first ten years of the 20th century. The basis of using the technique for pigments separation gave the technique its name. Development made during the 30s and 40s expanded the scope of the technique and became very useful for many separation processes [2]. In the 40s and 50s, two researchers Archer John Porter Martin and Richard Laurence Millington Synge developed the technique even further by establishing the principles and basic techniques of partition chromatography. Their contributions later won them the Nobel Prize in Chemistry in 1952 [3]. Their work sparked new life in the field by encouraging the rapid development of several chromatographic methods, including paper, gas, and high-performance liquid chromatography. Since the 21st century, technical performance of chromatography are now being advanced and improved continually and thus increasing further the scope of its use [4].

Overview and General Principle of Chromatography

Chromatography can be considered a ubiquitous technique in separation science, widely used in most laboratories and chemical process industry as a component of small and large-scale production. This is due to its versatility coupled with simplicity of approach and a reasonably well-developed framework in which the different chromatographic techniques operate. The method finds usefulness in analysis, isolation and purification of samples or substances.

Chromatography is basically a method of separation which involves three major components *via* stationary phase, mobile phase, separated molecules or substances. Stationary phase is always composed of a "solid" phase or "a layer of a liquid adsorbed on the surface a solid support", mobile phase is always composed of "liquid" (termed as liquid chromatography: LC) or a "gaseous component" (termed gas chromatography: GC) while separated molecules is substance separated by the interaction between the mobile and stationary phase. LC is used especially for thermal unstable, and non-volatile samples while GC is applied for gases, and mixtures of volatile liquids, and solid material [5,6]. Overall, the type of interaction between stationary phase, mobile phase, and substances contained in the mixture is the basic component effective on separation of molecules from each other. The separation is based on either by partitioning, adsorption, ion exchange and

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*Correspondence:
Enyoh Christian Ebere, Group Research in Analytical Chemistry, Environment and Climate Change (GRACE&CC), Department of Chemistry, Imo State University (IMSU), Imo State, Nigeria.
E-mail: cenyoh@gmail.com

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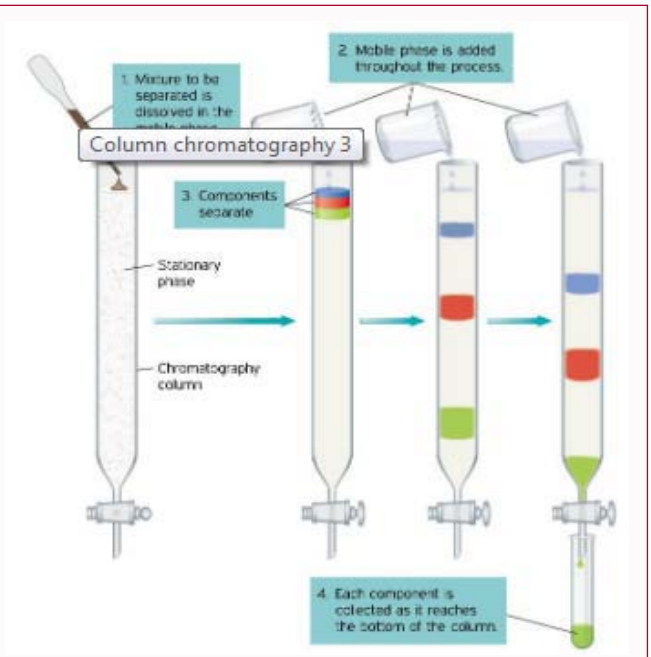

Figure 1: Application process for column chromatogprahic technique for separation [44].

size exclusion. For separation and identification of small molecules as amino acids, carbohydrates, and fatty acids, the partitioning basis is very effective while for large or macro molecules such as nucleic acid, and proteins the ion-exchange basis is more effective.

The purpose of applying chromatography which is used as a method of quantitative analysis apart from its separation is to achieve a satisfactory separation within a suitable time interval. Several key factors are responsible, therefore, or act together, to produce an acceptable separation [7]. Individual compounds are distinguished by their ability to participate in common intermolecular interactions in the two phases, which can generally be characterized by equilibrium constant, and is thus a property predicted from chemical thermodynamics. Interactions are mainly physical in type or involve weak chemical bonds, for example dipole-dipole, hydrogen bond formation, charge transfer, etc., and reversible, since useful separations only result if the compound spends some time in both phases [6].

The applications of chromatography are vast and often inter-disciplinary. So, various chromatography methods have been developed to that end. Some of them include column chromatography, Thin-Layer Chromatography (TLC), paper chromatography, gas chromatography, ion exchange chromatography, gel permeation chromatography, high-pressure liquid chromatography, and affinity chromatography. Ozlem [6] presented a short review on some of these techniques. Here, we extended his review focusing on applications of column, paper, thin layer and ion exchange chromatography in purifying samples.

Applications of Selected Chromatographic Techniques

Column chromatography (CC)

In column chromatography, the stationery phase is solid and the mobile phase is liquid. The column is prepared my mixing the silica with suitable solvent and poured in into a glass column. The packing can be achieved by generally two methods; dry and wet. In dry method at first the column is filled with dry powdered silica. Then the mobile phase, a suitable solvent is flushed through it until all the silica are wet and settled while the wet method a slurry of silica and solvent is first prepared and then poured onto the column using a funnel until the silica is settled into it. The application process for column chromatography is presented in Figure 1.

The compound mixture moves along with the mobile phase through stationery phase and separates depending on the different degree of adhesion (to the silica) of each component in the sample or the compound mixture. For example suppose a compound mixture contains three compounds blue, red and green (Figure 1). According to polarity, the order of these compounds were blue>red>green. That means blue is the most polar compound and thus will have fewer tendencies to move along with the mobile phase. The green colored compound will travel first as it is less polar than other two. When it is near end of the column a clean test tube is taken to collect the green sample. After this the red and at last the most polar blue compound is collected, all in separate test tubes. The samples movement through the stationery phase and accumulation at the bottom (through the outlet) of the device is time and volume-dependent [6,8] as well as polarity of the mobile phase.

The column chromatographic technique is one of the most convenient and widely used methods for purifying compounds. Often, synthetic reactions will produce multiple products and column chromatography can be used to isolate each of the compounds for further examination. Column chromatography is extremely valuable when synthesizing or isolating novel compounds, as very little needs to be known about a compound and its' physical properties prior to the purification process. The pharmaceutical industry routinely uses column chromatography to purify compounds as part of its early stage drug development process [9]. Often in these preliminary stages researchers will construct libraries of compounds around a lead compound, and then subsequently use column chromatography to purify the newly synthesized compounds [10].

Some other use is in the isolation and purification of compounds in plant during laboratory exercise. For example, Vivek et.al., [11] used the technique to isolate and purify plant secondary metabolites. The authors explained that the isolation of bioactive compounds using the column-chromatographic involves the following steps:

a) Preparation of sample

b) Packing of column

c) Pouring of sample into the column

d) Elution of fractions

e) Analysis of each fractions using thin layer chromatography

The isolation pattern obtained in the study is shown in Figure 2. They concluded that the techniques is very efficient to characterize both organic and inorganic materials and potentially useful in chemical analysis of complex extract material. They further assert that depending on nature of research, compounds can be further purified using High Performance Liquid Chromatography (HPLC), and Nuclear Magnetic Resonance (NMR) spectral analyses can be performed to elucidate the chemical structure of target compounds. Other studies in which the techniques have been applied include *Metasequoia glyptostroboides* Miki ex Hu (a deciduous conifer of the redwood family of Cupressaceae) [12,13].

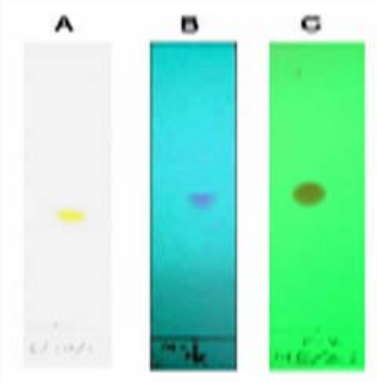

Figure 2: Demonstration of isolation pattern of pure compounds on TLC plate using iodine (A), UV-detection (B) and spray reagent (C) (Source: Vivek et. al., [11]).

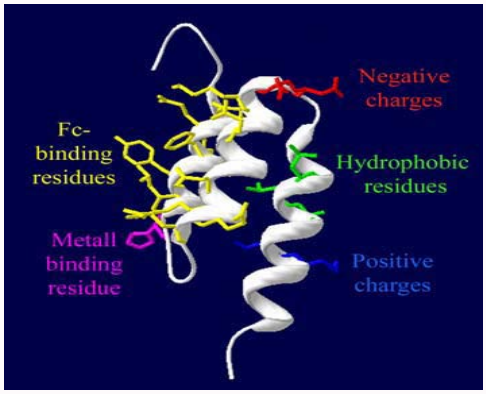

Figure 3: Some selective protein properties used to separate and purify one protein from another (Source: Hedhammar [15]).

Further use of column chromatography is in purifying proteins based on different features including size, shape, and net charge. Therefore the technique uses the chemical, biological and physical properties of the protein for its purification [14]. Some of the properties of protein used to separate one protein from another are shown in Figure 3 [15]. However, different proteins may require the use of different procedures and/or conditions. That is a procedure and condition used in the purification process of one protein may result in the inactivation of another. Furthermore, considerations based on desired purity and purpose is required when choosing a method for purification. In summary, column chromatography is a convenient and versatile method for purifying compounds. This method separates compounds based on polarity. By exploiting differences in the polarity of molecules, column chromatography can facilely separate compounds by the rate at which the compounds traverse through the stationary phase of the column. One of the benefits of column chromatography is that very little things about the compounds needs be known prior to the purification process. The other advantage to using column chromatography is that it can be used to purify both solids and oils. This technique can also be used to isolate a number of compounds from a mixture.

Paper chromatography (PC)

Paper chromatography is an analytical method used to separate colored chemicals or substances using a paper as the stationery phase (Figure 4). In paper chromatography support material consists

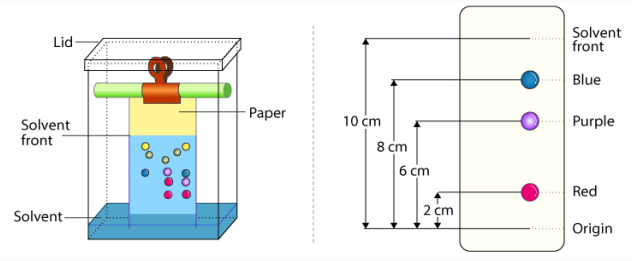

Figure 4: Principle of paper chromatography.

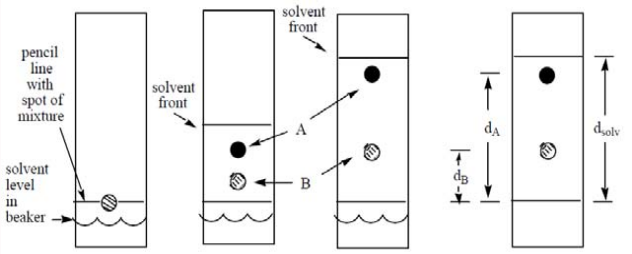

Figure 5: Paper chromatography - migration of two components.

of a layer of cellulose highly saturated with water. However, the characteristics of the paper can be changed by applying specific liquids to it. Silicone oils, paraffin oil, petroleum jelly, and rubber latex can be used to produce a paper with non polar liquid phases. Specially treated papers are also available, such as those containing ion-exchange resins. Papers for paper chromatography can also be made of glass fibers or nylon as well as cellulose [16]. Generally, in paper chromatography, a thick filter paper comprised the support, and water drops settled in its pores made up the stationary "liquid phase" while mobile phase consists of an appropriate fluid placed in a developing tank. Based on this, paper chromatography is often considered a "liquid-liquid" chromatographic technique.

The principle (Figure 4) involved in paper chromatography can be partition or adsorption chromatography. For partition, the substances are distributed between liquid phases while adsorption involves a solid (paper as stationery phase) and liquid phases (as the mobile phase).

Paper chromatography is an inexpensive analytical method and has been used to in purifying compounds with similar polarity such as amino acids [17-21]. For purifying amino acids, the paper used (could be a rectangular piece of filter paper) will be spotted at the bottom by applying amino acids solutions (very small volume). After the spotting process, the spotted paper will be rolled into a cylinder and placed in a beaker that contains a few milliliters of the liquid mobile phase (a solution containing n-propanol + water + ammonia). Immediately the paper is placed in the mobile phase, capillary action will occur, where the solution (eluting solvent) will begin to rise up the paper. The process will continue until the solution encounter the "spots" of amino acids on the stationery phase, where the purification or separation will occur [17].

The fate of each amino acid in the mixture now depends on the affinity of each substance for the mobile and stationary phases. If an amino acid has a higher affinity for the mobile phase than the stationary phase, it will tend to travel with the solvent front and be relatively unimpeded by the filter paper. In contrast, if the amino

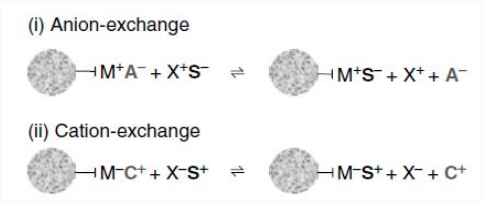

Figure 6: Ion-exchange chromatography schematic [31].

acid has a higher affinity for the paper than the solvent, it will tend to "stick" to the paper and travel more slowly than the solvent front. It is these differences in the amino acid affinities that lead to their separation on the paper. The affinities of these amino acids for the mobile phase can be correlated to the solubility of the different amino acids in the solvent. After the might have been removed and dried, it will be sprayed with ninhydrin (forms a blue-violet colored compound from reaction with amino acid), in other to make the amino acids on the paper visible by showing a number of spots, each one corresponding to an amino acid (e.g Figure 5). The further the spot from the starting line, the higher the affinity of the amino acid for the mobile phase and the faster its migration [17].

The relative extent to which solute molecules move in a chromatography experiment is indicated by R_f values. The R_f value for a component is defined as the ratio of the distance moved by that particular component divided by the distance moved by the solvent. Figure 5 represents the migration of two components. Measurements are made from the line on which the original samples were applied to the center of the migrated spot. In the figure, d_A is the distance traveled by component A, d_B is the distance traveled by component B, and d_{solv} is the distance traveled by the eluting solution. In all three cases, the travel time is the same. Thus, the R_f values for components A and B are $R_f(A) = d_A/d_{solv}$ and $R_f(B) = d_B/d_{solv}$ respectively .

Some studies in the 50's have applied this method in purifying amino acids mixtures [17-21] and also been applied for drug purification [22,23], plant extract purification and isolation for abscise acid [24] and isolation of cell wall teichoic acids of gram positive bacteria [25]. In summary, the technique is useful because it is relatively quick and requires only small quantities of material. However, drawbacks of this technique are based on its lengthy and time-consuming procedures with low resolving power and low reproducibility [26,27]. The use of paper chromatography is often replaced by the Thin Layer Chromatography (TLC) due to the two techniques operates by similar principle. However, PC is very effective in identifying unknown substances when samples are run on the same paper chromatograph with unknowns.

Thin layer chromatography (TLC)

Thin-layer chromatography is a "solid-liquid adsorption" chromatography. In this method stationary phase is a solid adsorbent substance coated on glass plates. Similar to PC principle, the mobile phase travels upward by capillary action through the stationary phase (thin plate soaked with the solvent). During this procedure, the mixture priory dropped on the lower parts of the plate with a pipette upwards with different flow rates. Thus the separation of analytes is achieved. This upward travelling rate depends on the polarity of the material, solid phase, and of the solvent. In cases where molecules of the sample are colorless, florescence, radioactivity or a specific chemical substance can be used to produce a visible colored reactive product so as to identify their positions on the chromatogram e.g. of such substance is ninhydrin or the use black-light visualization techniques. Formation of a visible color can be observed under room light or UV light. The position of each molecule in the mixture can be measured by calculating the ratio between the distances travelled by the molecule and the solvent. This measurement value is called relative mobility, and expressed with a symbol R_f. R_f value is used for qualitative description of the molecules [21].

TLC methodology is increasingly used in compounds purification such as amino acids, active ingredients, auxiliary substances and preservatives in drugs and drug preparations and also used in process control in synthetic manufacturing processes. It is also separation of multi component pharmaceutical formulations and vegetable drugs. TLC is also used to purify biological matrices for active substances and their metabolites e.g urinary constituent such as steroids, amino acids, porphyrins and bile acids. For purifying food samples for the determination of pesticides and fungicides and by using cationic and non-ionic surfactant-mediated systems as mobile phases, it has been used to purify aromatic amines on silica gel layers [28].

High sensitivity of TLC is used to check purity of sample, because high sensitivity enables impurities to be observed in so called pure samples [18,29]. Thin layer chromatography is simple, cost-effective, and easy-to-operate technique in analytical chemistry with numerous applications which use in the development of new drugs and various types of formulations from medicinal plants. Thin Layer Chromatography (TLC) is now also called planar chromatography [30].

Ion exchange chromatography (IEC)

Like all other chromatography techniques discussed, IEC technique also involves a mobile and stationary phase, in which separation of molecules is based on charge. The stationary phase here is a column packed with ion exchange resins while the mobile phase is typically an aqueous buffer system into which the mixture to be resolved is introduced. In the system are counter ions which are in a state of equilibrium between the two phases [31], giving rise to two possible IEC formats, namely anion- and cation-exchange (see Figure 6). Those resin that echange cations are called catex while those that exchange anions are called annex. Exchangeable matrix counter ions may include protons (H^+), hydroxide groups (OH^-), single charged monoatomic ions (Na^+, K^+, Cl^-), double charged mono atomic ions (Ca^{2+}, Mg^{2+}), and polyatomic inorganic ions (SO_4^{2-}, PO_4^{3-}), as well as organic bases (NR_2H^+) and acids (COO^-) [31].

Figure 6 Ion-exchange chromatography schematic. It is the nature of the counter ions displaced from the matrix functional groups (M^+, M^-) which determines the IEC format. Thus, with anex, the stationary phase (usually a porous bead) displays a positively charged functional group with counter anion (A^-) that can be displaced by an anionic solute (S^-). By contrast, with catex, the stationary phase displays a negatively charged functional group with counter cation (C^+) that can be displaced by a cationic solute (S^+) [31].

Ion exchange is the most widely used chromatographic method for the separation and purification of charged bio molecules such as polypeptides, proteins, poly nucleotides, and nucleic acids [32-43]. Some industrial applications of the technique including: the separation and purification of blood components such as albumin, recombinant growth factors and enzymes, in studying wheat varieties and the correlation of proteinuria with different renal diseases and

the cation exchange resins are used to monitor the fermentation process during β-galactosidase production [41-43]. The key steps in the ion exchange chromatography procedure are listed below:

1. An impure protein sample is loaded into the ion exchange chromatography column at a particular pH.

2. Charged proteins will bind to the oppositely charged functional groups in the resin.

3. A salt gradient is used to elute separated proteins. At low salt concentrations, proteins having few charged groups are eluted and at higher salt concentrations, proteins with several charged groups are eluted.

4. Unwanted proteins and impurities are removed by washing the column.

A pH gradient can also be applied to elute individual proteins on the basis of their isoelectric point (pI) i.e. the point at which the amino acids in a protein carry neutral charge and hence do not migrate in an electric field. As amino acids are zwitter ionic compounds they contain groups having both positive and negative charges. Based on the pH of the environment, proteins carry a positive, negative, or nil charge. At their isoelectric point, they will not interact with the charged moieties in the column resin and hence are eluted. A decreasing pH gradient can be used to elute proteins using an anion exchange resin and an increasing pH gradient can be used to elute proteins from cation exchange resins. This is because increasing the buffer pH of the mobile phase causes the protein to become less protonated (less positively charged) so it cannot form an ionic interaction with the negatively charged resin, allowing is elution. Conversely, lowering the pH of the mobile phase will cause the molecule to become more protonated less negatively charged, allowing its elution [21,31,38].

IEC applicability is widespread, large sample-handling capacity, high capacity and simplicity, and its high resolution applicability (including high performance and high-throughput application formats), broad moderate cost, powerful resolving ability, and ease of scale-up and automation are the key reasons for its success as a separation method and have led to it becoming one of the most versatile and widely used of all liquid chromatography techniques [31].

Conclusion

Following the first use of chromatography by Mikhail Tsvet in 1900, as with the case of herbal pigment, the technique was used initially to separate substances based on their color. It applications are now wide spread and it is extremely sensitive, and effective purification method especially for protein molecules, nucleic acids, drugs, antibiotics and biological matrices. Finally, these techniques does not require the use of machines or special devices, it is fully portable and easy to handle and considerably cheaper than most commercial methods.

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