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Separation, purification and diagnosis of more than one biological active compounds from Ginger rhizomes using HPLC and LCMS

A graduation project is submitted to clinical lab sciences department at college of pharmacy in partial fulfillment of the requirement for the degree of Bachelor of Science

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بِسْمِ اللَّـهِ الرَّحْمَـٰنِ الرَّحِيمِ

(يرفع الله الذين آمنوا منكم والذين أوتوا العلم درجات

والله بما تعملون خبير)

صدق الله العلي العظيم

(سورة المجادلة:11)

Certification of the Supervisor

I certify that this project entitled "Separation, purification and diagnosis of more than one biological active compounds from Ginger rhizomes using HPLC and LCMS"

was prepared by the fifth-year students : Aya Abdulkareem Hussein

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under my supervision at the Department of clinical lab sciencesin at University of Basra /college of pharmacy partial fulfilment of the graduation requirements for the bachelor’s degree in pharmacy.

Dr.Haider A. Alwafi

Signature:

Dedication

To express our thanks to all those who contributed in many ways to the success of this study and made it an unforgettable experience for us.

To Our God Almighty who is always there when we are in need.Thank you for guiding us and giving strength in our everyday life.

To our dear parents, Thank you for giving me the support to reach my dreams. Accomplishing this would hopefully make you proud of me as much as I am proud of having you as my parents.

We would like to sincerely thank our Supervise,Dr.Haider A. Alwafi for your guidance, support, and patience throughout this study, we apologize for being a headache to you when we was doing this study.

1-Introduction

Ginger (Zingiber officinale Roscoe), a well-known herbaceous plant, has been widely used as a flavoring agent and herbal medicine for centuries. Furthermore, the consumption of the ginger rhizome is a typical traditional remedy to relieve common health problems, including pain, nausea, and vomiting. Notably, a prominent number of randomized clinical trials (RCTs) have been conducted to examine ginger's antiemetic effect in various conditions such as motion sickness, pregnancy, and post-anesthesia [1].

Ginger also has been used as a medicine for treating diabetes, flatulent intestinal colic, indigestion, infertility, inflammation, insomnia, a memory booster, nausea, rheumatism, stomach ache, and urinary tract infections [2]. Moreover, in recent years, the role of ginger has been extended to anticancer, chemotherapy-induced nausea and vomiting (CINV), and fatigue, as well as improvements in the quality of life in daily human work [1]. Also, Ulcerative colitis, Crohn's disease, rheumatoid arthritis, psoriasis, and lupus erythematosus are some of common inflammatory diseases. These affections are highly disabling and share signals such as inflammatory sequences and immune dysregulation. The use of foods with anti-inflammatory properties such as ginger (Zingiber officinale Roscoe) could improve the quality of life of these patients [3].

Furthermore, there are more than approximately 400 compounds have reportedly been isolated from ginger. Specifically, the major classes of ginger compounds are gingerol, shogaols, zingiberene, zingerone, diarylheptanoids, gingerol analogues, phenylalkanoids, sulfonates, monoterpenoid glycosides, steroids, and terpene, as well as other less common compounds, including terpenes, vitamins, and minerals [1,2].

Chemical standardization of these products is needed for quality control and to facilitate the design of clinical trials and the evaluation of data from these studies. To address this issue, methods based on liquid chromatography mass spectrometry (LC-MS) were developed for the detection, characterization and quantitative analysis of gingerol-related compounds in botanical dietary supplements containing ginger roots/rhizomes [4].

Moreover, several high-performance liquid chromatographic (HPLC) methods have been developed for the analysis of ginger extracts. These methods were primarily used for the analysis of gingerols from field-grown ginger, but not for the analysis of gingerols from in vitro- grown ginger, which is now being explored as a potential alternative source of [6]-gingerol. Recently, an HPLC method for the analysis of gingerols from both the field-grown rhizomes and in vitro-grown micro rhizomes of ginger has been developed. However, this method involves several extra steps (e.g. ethyl acetate extraction, drying over anhydrous sodium sulfate and filtering), which could be prone to error due to losses associated with these extra steps [5].

Ginger is used in numerous forms, including fresh, dried, pickled, preserved, crystallized, candied, and powdered or ground. Presentations can include capsules, tablets as in figure(1) , tinctures, teas, and liquid extracts. Evidently, the concentrations of active ingredients (gingerols and shogaols) will differ between the different preparations and the processing steps involved. Indeed, gingerols are thermally labile, and the extent of gingerol-to-shogaol conversion will likely impact significantly on the medicinal benefits since the two compounds vary in their bioavailability and pharmacological properties.

Figure (1)

A recent methodological analysis using high-performance liquid chromatography (HPLC) coupled to time-of-flight mass spectrometry demonstrated that dried ginger powder products contained the highest quantity of gingerol-related compounds (7–14 mg/g), followed by fresh ginger (2–2.8 mg/g) and powdered ginger tea products (~0.8 mg/g). Attempts to assess the efficacy of ginger in many clinical trials might have been conceivably weakened by the inconsistency in the form of ginger used (fresh or dried) and also the dosing regimen [6].

After the process of extraction, the chromatographic method is used as technique to save the environment with more accuracy rate, precise and reproducible conclusions such as fast pressure column, gas chromatography and liquid chromatography. Each of these techniques is reliable on the nature of elucidated compound by keeping in view the sensitivity of the instrument. For this purpose, high performance liquid chromatography (HPLC) is a conclusive instrument for the quantification and characterization of bioactive components present in the extracts of different parts of ginger. Different parameters are used in HPLC depending upon the assorted difference in the structure of bioactive entities extracted in extracts such as column temperature, type of detector used, pressure difference as well as wavelength [7].

HPLC (High-performance liquid chromatography or high-pressure liquid chromatography) is basically a highly improved form of column chromatography. It is a technique in analytical chemistry used to separate, identify and quantify(qualitative and quantitative analysis) each component in a mixture.

HPLC is distinguished from traditional (low pressure) liquid chromatography because operational pressures are significantly higher (50–400 bar), while ordinary liquid chromatography typically relies on the force of gravity to pass the mobile phase through the column Figure2:

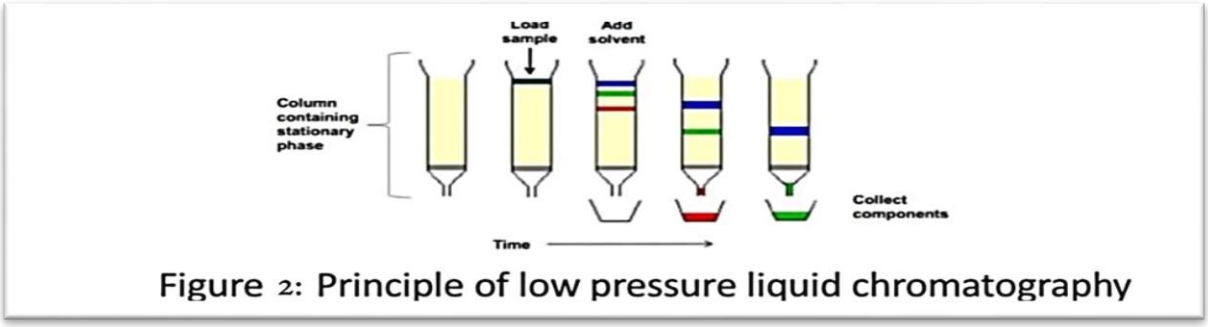

Figure 2: Principle of low pressure liquid chromatography

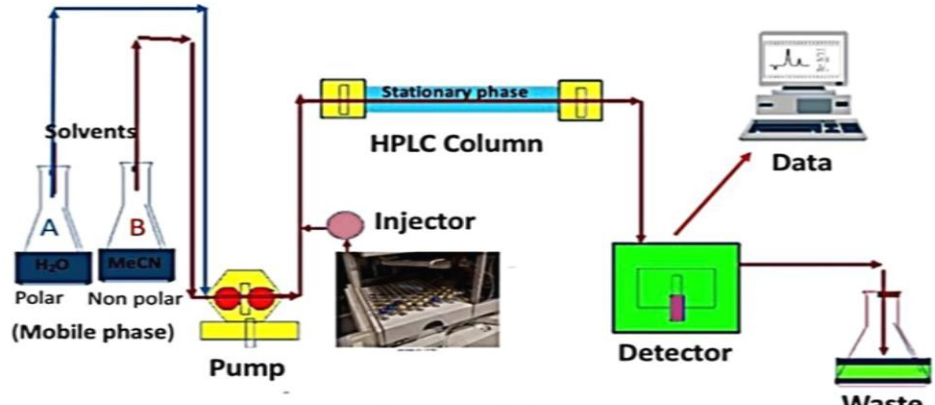

Figure 3: Diagram of HPLC system

There are two classes of HPLC technique:

1-Normal phase HPLC(NP-HPLC): The column is filled with tiny silica particles(polar material), and the solvent is non-polar-hexane, for example. A typical column has an internal diameter of 4.6 mm (and may be less than that), and a length of 150 to 250 mm.

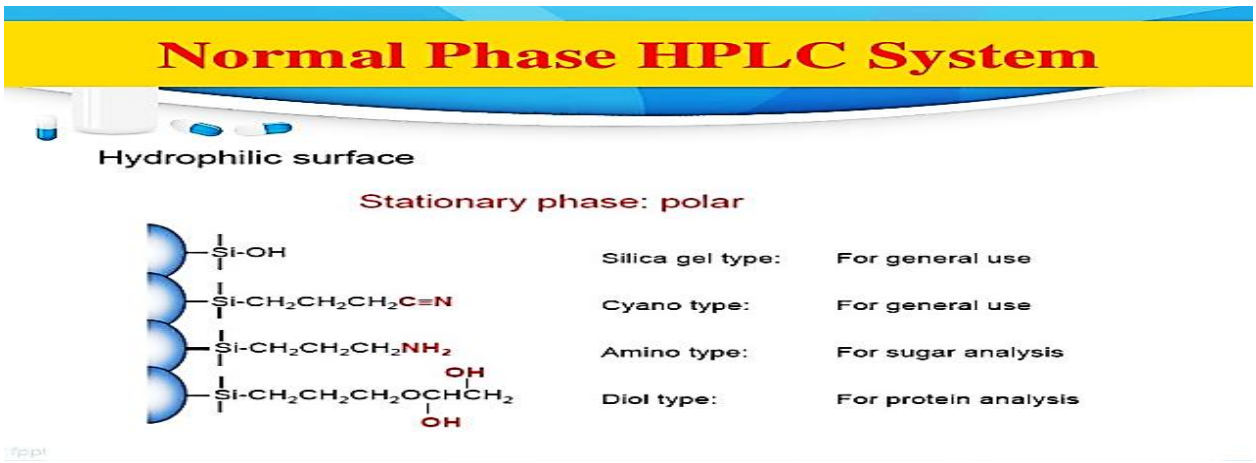

Figure 4: Types of stationary phase in normal phase HPLC system

Polar compounds in the mixture being passed through the column will stick longer to the polar silica than non-polar compounds. The non-polar ones will therefore pass more quickly through the column.

Example for the normal phase is thin layer chromatography (TLC) or column chromatography. Although it is described as "normal", it is slow and less accuracy technique.

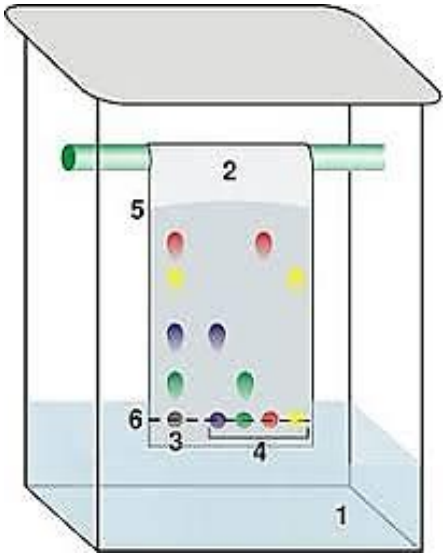

Figure 5: TLC chromatography

2-Reversed-phase HPLC: In this case, the column size is the same, but the silica is modified to make it non-polar by attaching long hydrocarbon chains to its surface, typically with either 8 or 18 carbon atoms in them. A polar solvent is used - for example, a mixture of water and an alcohol such as methanol or acetonitrile.

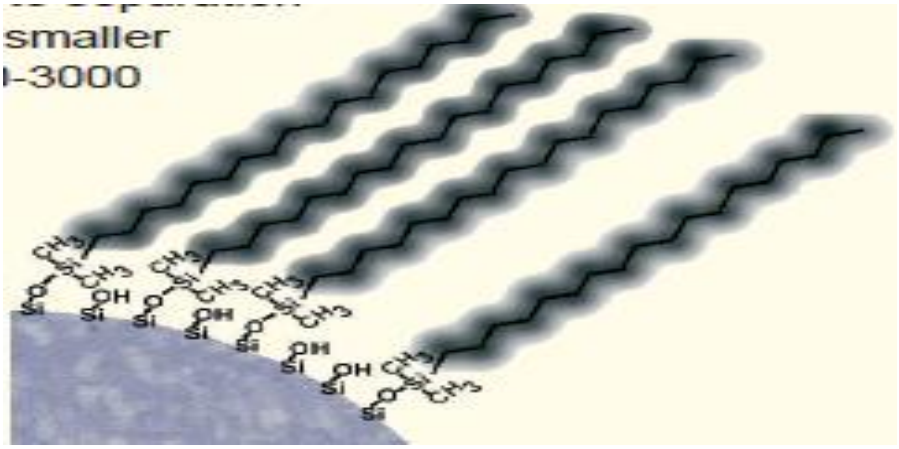

Figure 6: Non-polar stationary phase (C_{18}) inreversed-phase HPLC

In this case, there will be a strong attraction between the polar solvent and polar molecules in the mixture being passed through the column. Polar molecules in the mixture will therefore **spend most of their time moving with the solvent** that means, the polar molecules will be travelled through the column more quickly.

Non-polar compounds in the mixture will tend to form attractions with the hydrocarbon groups because of van der Waals dispersion forces. Therefore, non polar molecules in the mixture will spend most of their time attracting with the stationary phase leading to late elution from the column.

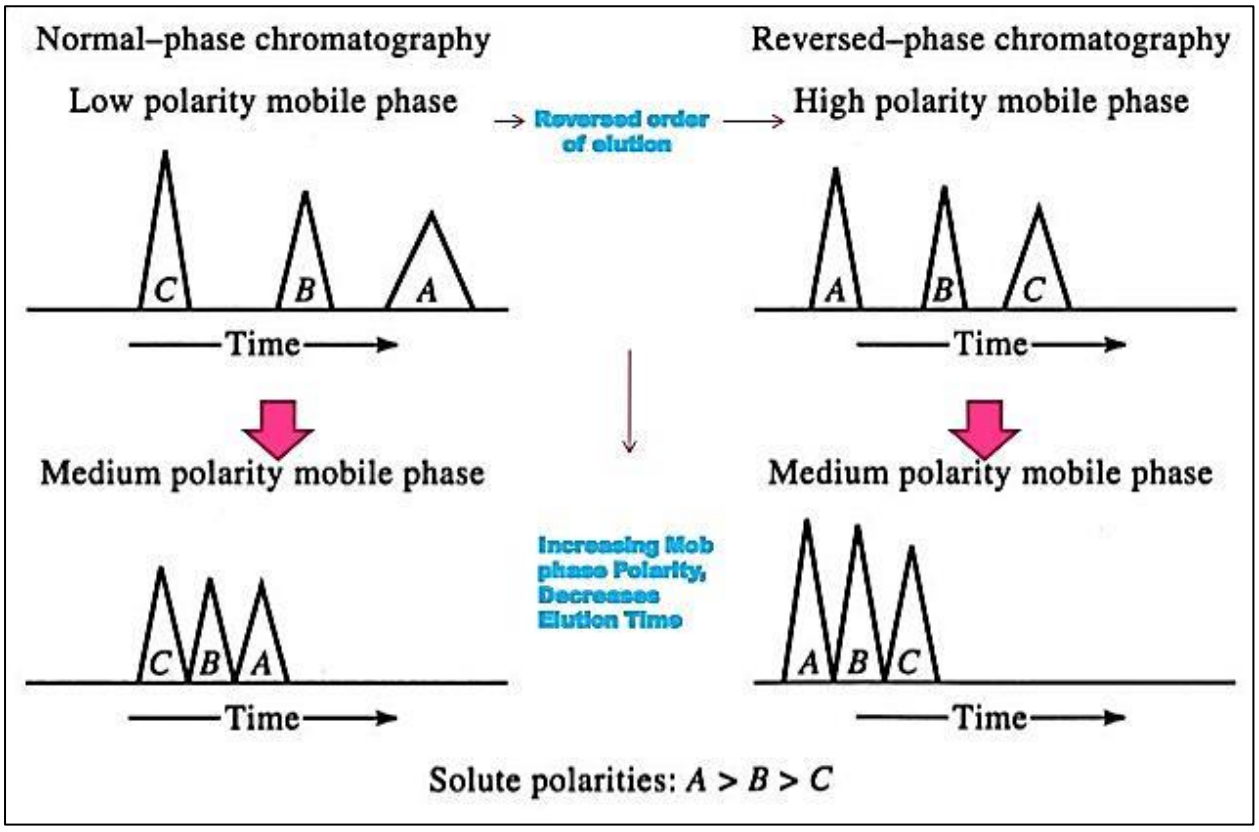

Figure 7: The relationship between polarity and elution times for normal phase and reversed-phase chromatography

As a conclusion, the principle of the separation by reversed-phase HPLC relays on adsorption of the compounds on surface of the stationary phase then will be separated (eluted) according to the range of their affinity to make attractions. Strong binding means longer retention time.

Some of the important factors affected on the separation by RP-HPLC

1- Particles: The particles in the column are usually made of silica because silica is physically robust, it is stable under most solvent conditions (except at pH greater than 6.5) and silica can be made into spherical particles of various sizes.

2-Pore Diameter: Using small pore (~100 angstrom) silica normally used in reversed phase HPLC results in inferior protein separations. Wide pore silica (~300 angstrom diameter) gives much better separations of proteins

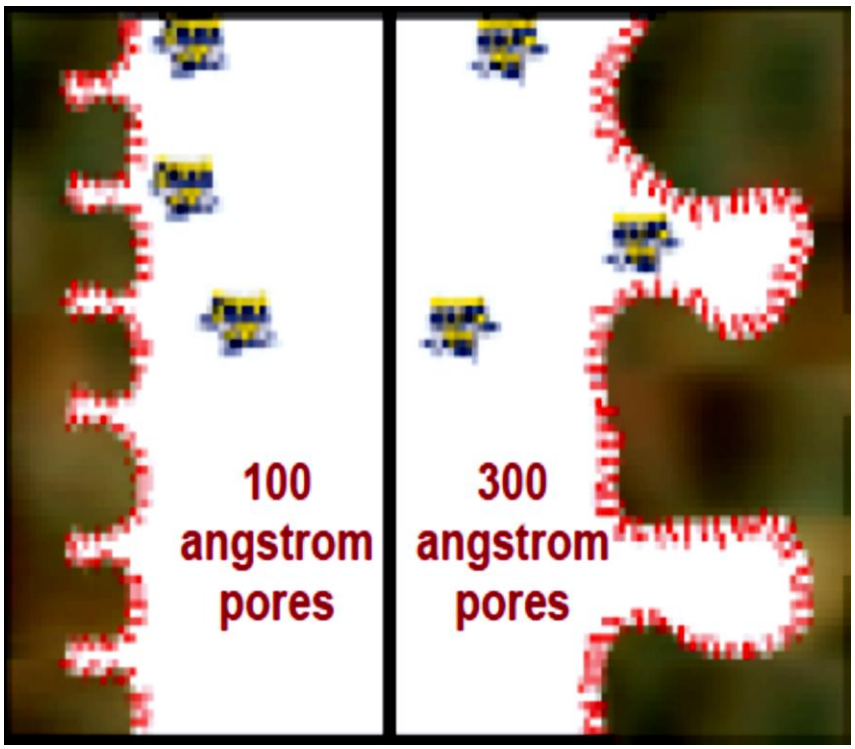

Figure 8: limit surface interaction in the left (small pore particles). More surface interaction in the right(particles with wide pores).

3-Purity of the silica: The purity of the silica used in HPLC columns is important in separation performance by RP-HPLC.

A. Lower purity silica:

Vydac C18 , 4.6 x 250 mm

B. High purity silica:

ACE 5 C18 , 4.6 x 250 mm

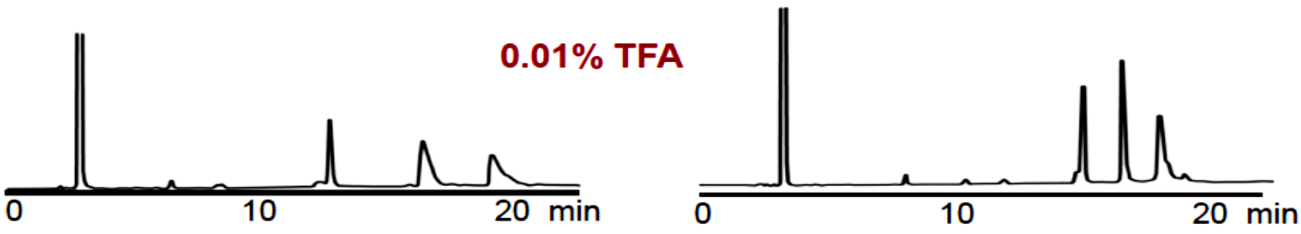

Figure 9: Effect purity of the silica on the separation by RP-HPLC

4- Hydrophobic surface: The silica is modified with a hydrocarbon molecule in order to create a hydrophobic surface.

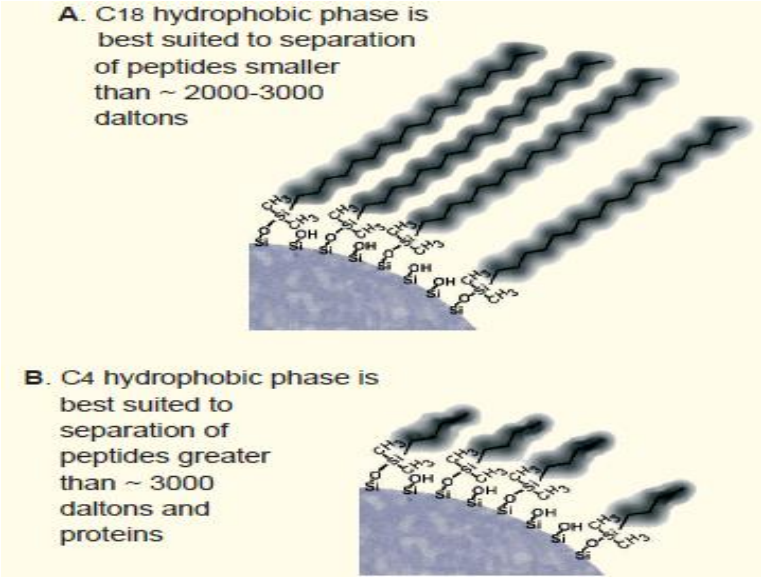

Figure 10: Effect of the hydrophobic surface on the separation by RP-HPLC

5-Column length: Long columns give higher resolution than short columns for small molecules such as small peptides. But Column length is not important in protein separations and short columns separate proteins as well as long columns.

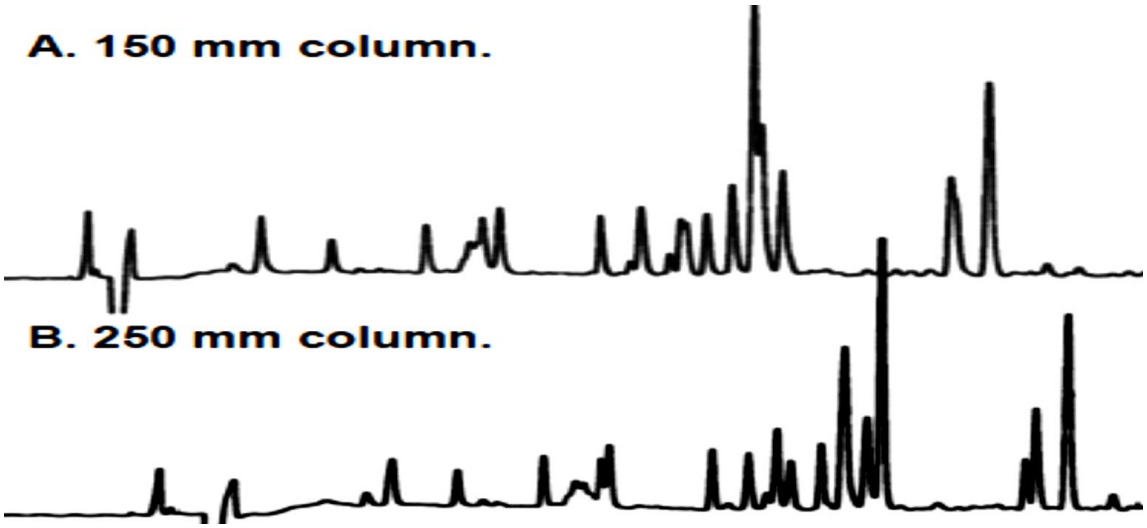

Figure 11: separation of the protein using different length column

Proteins adsorb and desorb near the top of the column. Once desorbed, proteins interact minimally with the hydrophobic phase, thus longer columns do not increase resolution with proteins as they do with small molecules. Fifteen or twenty-five centimetre columns are recommended for peptide separations.

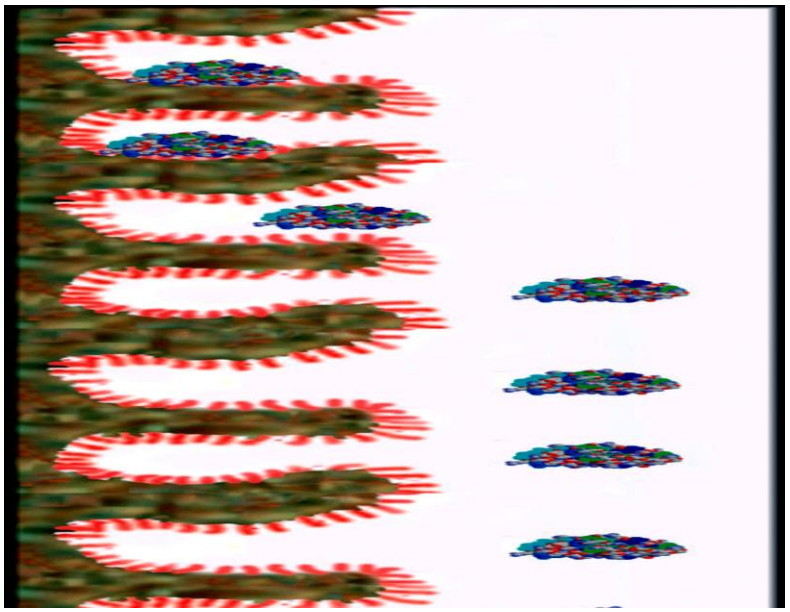

Figure 12: Separation of the proteins at the top of the column.

6-Column Diameter: The standard diameter of analytical HPLC columns is 4.6 mm. These columns are best run at flow rates of ~1 ml/min**.**

Narrow bore columns also exhibit about five times the sensitivity of standard analytical columns. Micro bore columns operate at flow rates of ~50 microlitres/minutes and therefore their use results in even better sensitivity, about 50 times that of an analytical column. Finally, capillary columns operating at 1 - 50 microlitres/minute flow rates exhibit even higher relative sensitivity, about 200 times the sensitivity of an analytical column.

Figure 13. Characteristics of columns of different diameters

Column type	Analytical	Narrow Bore	Microbore	Capillary
Diameter	4.6 mm	2.0 mm	1.0 mm	< 1 mm
Flow rate	~ 1 ml/min	~ 200µL/min	~ 50 µL/min	5 - 50 µL/min
Relative sensitivity	1	5	50	200

Figure 13: Characteristics of columns of different diameters

7-Gradient elution: A reduction of the gradient slope to improve resolution must be tempered with the need for keeping analysis time as short as possible. However, adjusting the gradient slope is important in optimizing resolution of proteins and peptides.

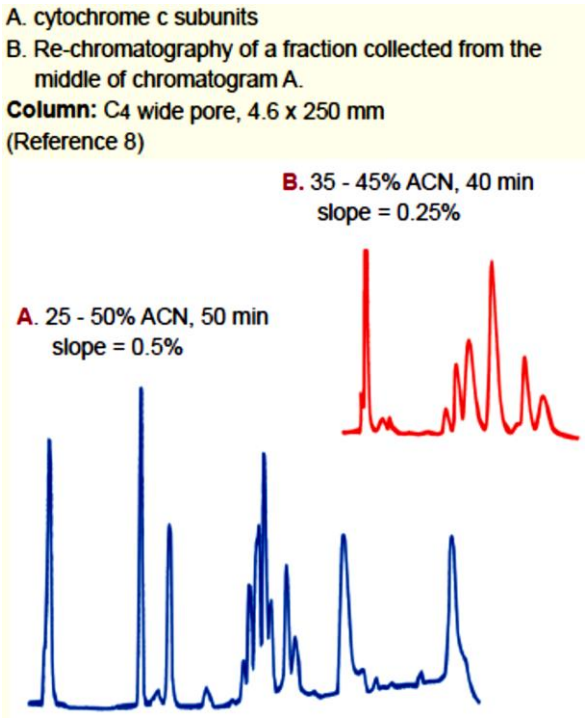

Figure 14: Reducing the rate of change of organic solvent in the gradient generally improves resolution.

8-Ion-pair reagent: The most commonly used ion-pair reagent is trifluoroacetic acid (TFA). TFA added to the mobile phase at a concentration of ~0.1% results in good peak shape on most columns.

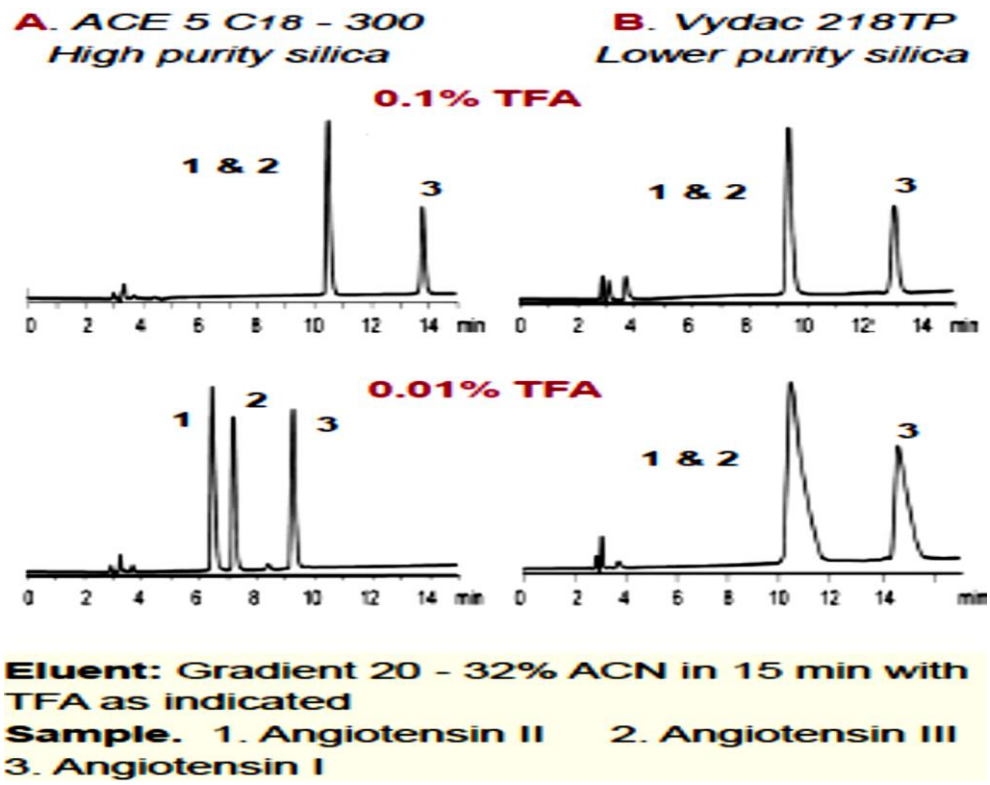

Figure 15: The effect of TFA concentrations on peak shape and selectivity

2-Experimental

2.1 preparation of the crude sample

1. Ginger was bought from the local market.

2. (10gm) of the ginger was scratched by the scratcher for getting the paste form.

3. Then the paste form was put in the beaker flask of the freezing dryer and stored in the freezer at (-20 ^{o}C) for 24 hours.

Figure 2-1: Crude sample in freezing dryer containers

4. Then the frozen sample was connected to the freezing dryer under temperature (-52 °C) and pressure (0.15 mbar) for 48 hr.as in Figure (2.2).

Figure 2.2: frozen crude ginger sample is connected to the freeze dryer

5. The sample was dried 99%.

6. The sample was milled by the grinder for getting very soft powder.

Figure2-3: powder of crude ginger sample

7 (100 mg) of soft crude sample was weighted for five times.

8. Each one was dissolved according to the following Table (2.1).

Table 2.1: amount and type of solvents that used to prepare the samples

Ginger powder	W	M	A	W+A	M+A
1-100 mg	100%(1ml)				
2-100 mg		100%			
3-100mg			100%		
4-100 mg				50%+50%	
5-100 mg					50%+50%

W=water, M=methanol, A= acetonitrile

9. Then the sample was put in the ultrasonic bath or for 2min after 2 min mixing by the vortex.

10. The vials was centrifuged for 3min at 6000 rpm.

11. The clear solution was transferred using micropipette to the HPLC vial to be ready to run.

figure 2-4: different samples

3-Results

Figure 3-1 indicates HPLC chromatogram of crude Ginger sample (water extracted). There are two clear overlap peaeks, the first is sharp and high intensity peak with retention time at 2.6 min. While the second is also sharp and high intensity peak but less than the first, at the retention time 3.2 min. The presence of thses compounds at low retention time (less than 5 min) and very close together indicates that these compounds have about very close polarity therefore they are separated at very close low retention times (2.6-3.4) min. In addition to that, these compound may have close molecular weights.

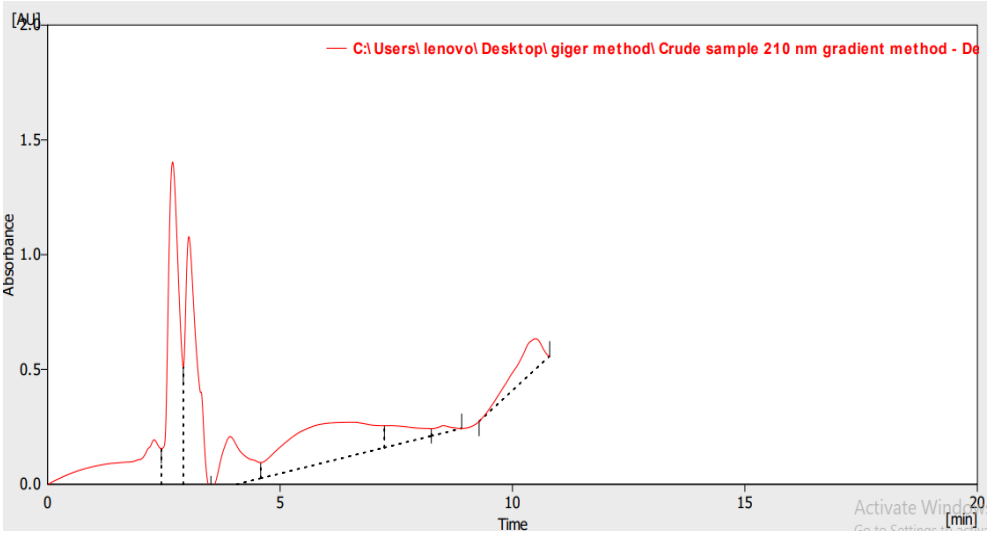

Figure 3-1: HPLC chromatogram of crude ginger sample (water extracted)

Figure 3.2 represents HPLC chromatogram of purified compound at retention time 2.6 min for the water extracted crude sample. The high intensity sharp peak at the same retention time (2.6 min) as in crude sample (Figure 3.1) clarifies that the used method to separate and purify this compound was successful despite there is some very low intensity broad peaks at before and after the retention time of the compound's peak (2.6 min) which could be removed by decrease the injection volume of the sample. So the first peak was separated and collected successfully with high purity. The purity is increased with using less injection volume for the crude sample.

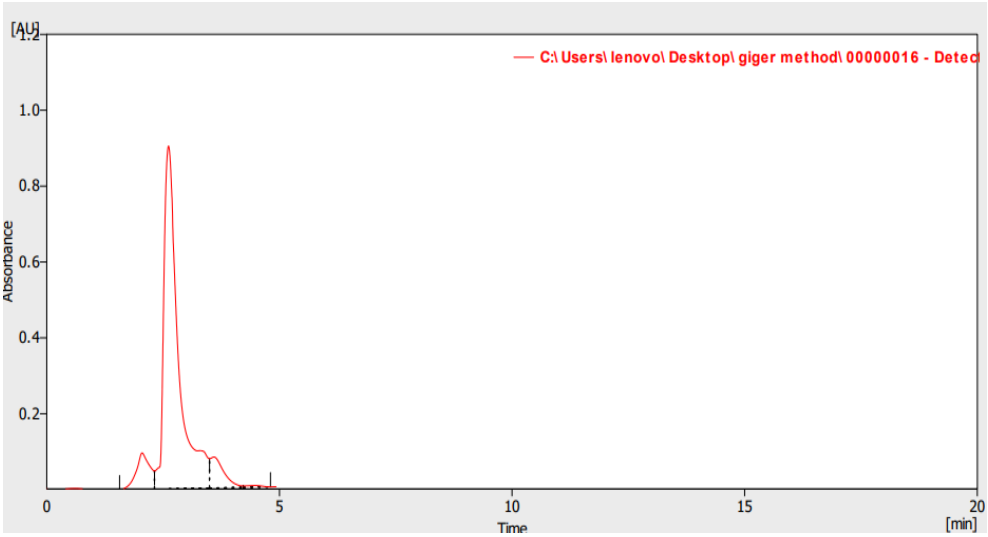

Figure 3-2: HPLC chromatogram of purified compound at retention time 2.6 min (water extracted)

Figure 3.3 clarifies the HPLC chromatogram of purified compound at the retention time 3.1 min for the water extracted of the crude sample). The peak at the retention time 3.1 min is the same peak in the chromatogram of crude sample (Figure 3.1). the property of this peak is single, high and without overlap with other peaks as the concentration of the others are low so, it easy to remove the others using less injection volume. This result is indicator for successful method for the separation and, purification and collection of the compound.

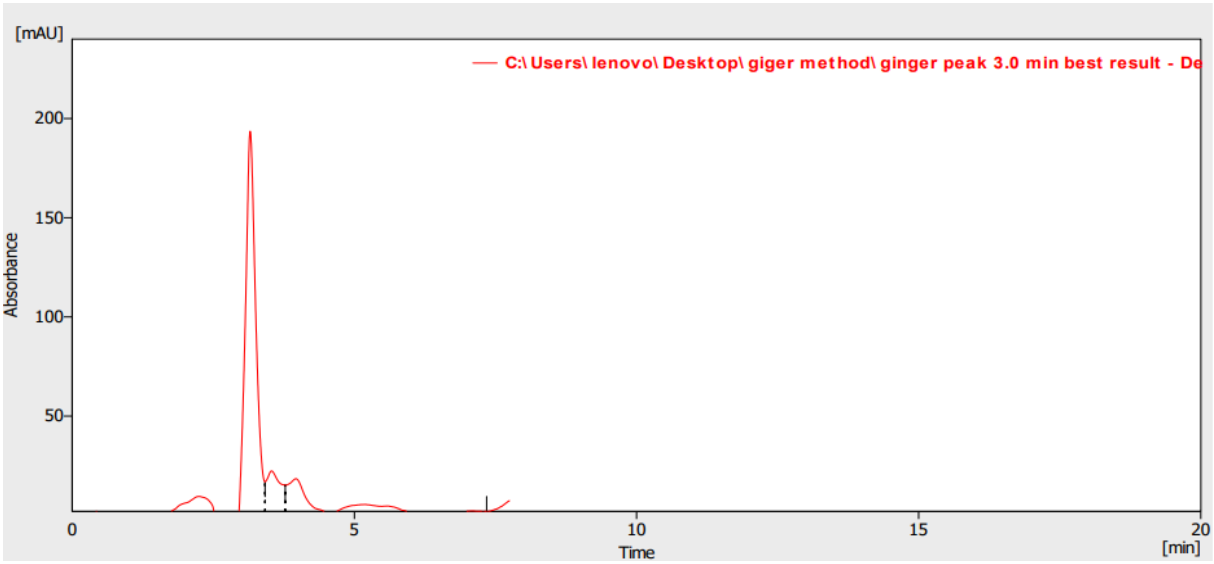

Figure 3-3: HPLC chromatogram of purified compound at the retention time 3.1 min (water extracted)

The following chromatogram (Figure 3.4) shows HPLC chromatogram of crud ginger sample (acetonitrile extracted). The method was more successful to separate the two compounds after using acetonitrile as a solvent to dissolve the solid crude ginger sample, the retention time of the first peak was less than 5 min. while, the second was more than 20 min. so, the collection each compound as a lone is very easy without repeating the collection step of the same peak.

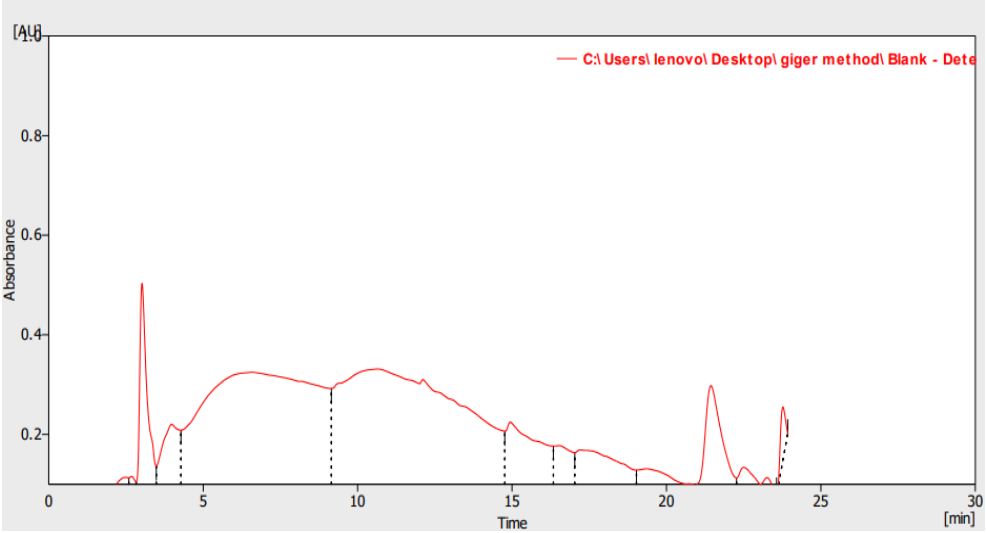

Figure 3-4: HPLC chromatogram of crud ginger sample (acetonitrile extracted)

4-Conclusion

It was a successful method to separate two pure compound from crude ginger sample by using more than one solvent .

5-References

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