Republic of Iraq

Ministry of higher education and scientific research

University of Basra/College of pharmacy

Separation and purification of two biological active compounds from powder curcumin using HPLC.

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

By:

Shahad Ahmed Ramadhan. **Manar Mohammed Amen**

Mariam Ali Zaydan

Supervised by:

Dr. Haider A. Alwafi

اقْرَأْ بِاسْمِ رَبِّكَ الَّذِي خَلَقَ (١) خَلَقَ الإِنسَانَ مِنْ عَلَقٍ (٢) اقْرَأْ وَرَبُّكَ الأَكْرَمُ (٣)

الَّذِي عَلَّمَ بِالْقَلَمِ (٤) عَلَّمَ الإِنسَانَ مَا لَمْ يَعْلَمْ (٥) سورة العلق

صدق الله العظيم

Dedication

To everyone that was part of our journey in preparing this project and contributed in finishing it , a family , a friend and a guider ..

But first of all thanks God for the great grace that He has bestowed upon us, for all our professors who supported us in our journey in the College of Pharmacy. It was an unforgettable experience. I am also pleased to thank the esteemed college administration Basra university (college of pharmacy).

Thanks to Dr. Haider A. Alwafi for his valuable time for helping us to complete our project ,guided and directed us in preparing this research and for connecting us to the required references and sources.

We hope to be at everyone's expectations..

Certification of the Supervisor

I certify that this project entitled "Separation and purification of more than one biological active compounds from alcoholic extracted of curcumin using HPLC

was prepared by the fifth-year students :

Manar Mohammed Ameen Hashim

Shahid Ahmed Ramadhan

Mariam Ali Zaidan

At the Department of clinical lab sciences in the University of Basra /college of pharmacy partial fulfilment of the graduation requirements for the bachelor’s degree in the sciences of pharmacy.

Dr.Haider A. Alwafi

1-Introduction:

Curcumin, also known as diferuloymethane, is a hydrophobic polyphenol derived from the rhizome of perennial herbs genus Curcuma which belongs to the ginger family (Zingiberaceae) and includes species like Curcuma longa, Curcuma amada, Curcuma zedoaria, Curcuma aromatic, Curcuma raktakanta.

Among these species, Curcuma longa (turmeric) is the most popular. Generally, turmeric rhizomes contain 3–5% of three types of curcuminoid derivatives including curcumin (75%), demethoxycurcumin (10–20%) and bisdemethoxycurcumin (5%), curcumin being the most important bioactive compound Curcumin has long been used as a spice and a natural coloring agent in Indian curries, as well as a component of Chinese traditional medicines.

As a food additive, its E number (codes for substances used as food additives for use within the European Union (EU) and European Free Trade Association (EFTA)) is E100.

In recent years, numerous in vivo and in vitro studies have revealed that curcumin has various physiologic activities, and worldwide attention has therefore been focused on curcumin. Curcumin has been found to have multiple actions such as anti-oxidant through scavenging of free radicals, antiinflammatory by suppression of NF-kB and AP-1 activation, anticancer through inhibition of cancer cell proliferation, induction of apoptosis, suppression of angiogenesis, inhibition of the expression of anti-apoptotic proteins, protection of the immune system, and anti-bacterial/fungal/viral activities.

Due to its wide range of pharmacological activities, curcumin shows potential for commercial use in different industries including food, cosmetics and pharmaceutics.[1]

There are two methods for obtaining curcumin: by means of synthesis and by extraction from plants. Many authors have described the preparation of synthetic curcumin.

Nevertheless, obtaining curcumin, which is naturally present in plants by means of extraction still represents the most economical way for curcumin production.

To date, various extraction techniques ranging from conventional (e.g. soxhlet extraction, maceration and solvent extraction) to advanced extraction technologies (e.g. ultrasound-assisted extraction, microwave-assisted extraction, enzyme-assisted extraction, supercritical liquid extraction, etc.)[2].

It is stated that the extraction procedure plays a critical role in determining the quantity and quality of bioactive compounds.

In order to obtain high yield and high quality curcumin, it is very important to choose appropriate and effective methods and operate these under optimal conditions[1].

Mainly these activities of curcuminoids are due to the presence of three structurally correlated curcuminoids, viz. 1,7-bis(4- hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione (Curcumin, C) 1-(4-hydroxy phenyl)-7-(4-hydroxy-3- methoxyphenyl)-1,6-heptadiene-3,5-dione (demethoxy curcumin, DMC) and 1,7-bis(4-hydroxyphenyl)-1,6-heptadiene-3,5-dione(bis-demethoxycurcu min, BDMC).

The anticancer activities order of these curcuminoids is BDMC > DMC > C." The structures of these curcuminoids are given in Figure 1, indicating two substituted phenyl rings separated by heptadienone spacer.

The commercially available turmeric powder is a mixture of naturally occurring curcuminoids; with curcumin (C) as the major constituent with other two (DMC and BDMC) as 2,12 minors (C: DMC: BDMC: 77: 18: 5%).

Due to the different pharmaceutical properties of three curcuminoids, their separation and identification are important issues in medicinal chemistry[3].

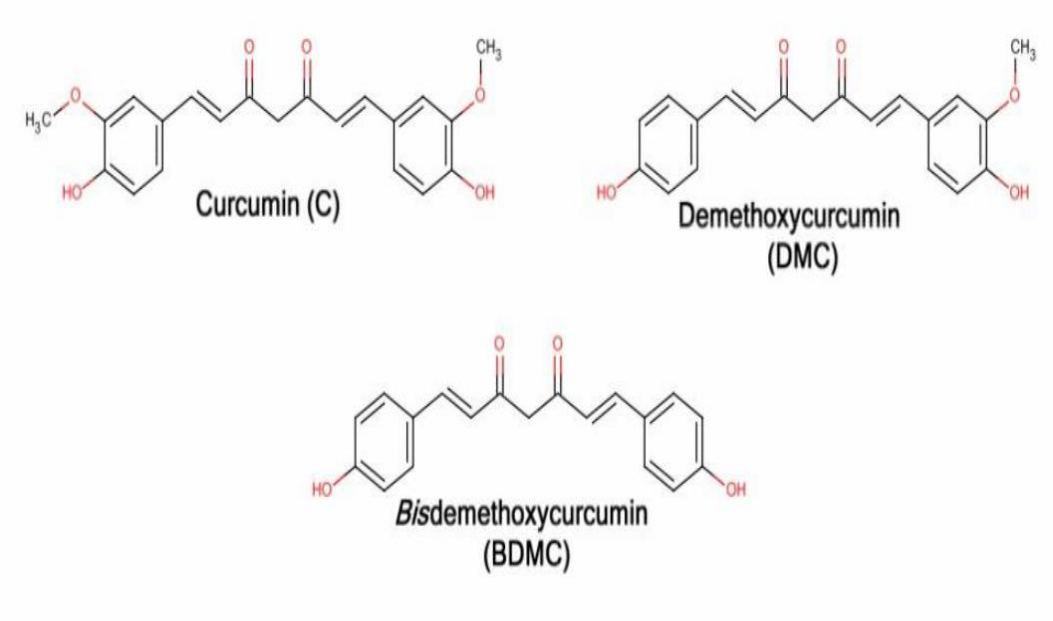


Figure 1 : structures of curcuminoid

Consequently, many different purification techniques, alone or in combination, have been exploited to isolate and purify curcumin from curcumin extracts or commercially available crude curcumin.

Conventional purification techniques (e.g. column chromatography, semi-preparative high performance liquid chromatography) as well as supplementary modern techniques (e.g. high-speed counter-current chromatography, supercritical fluid chromatography, etc.) have been used to separate and purify curcumin.

Obtaining curcumin from plant sources is very important for fundamental research, and for practical applications in food, cosmetics and pharmaceutical fields. However, its application has remained challenging due to some of its properties, i.e., poor water solubility, chemical instability, photo degradation, relatively high rate of metabolic degradation, and low bioavailability.

Therefore, numerous studies have attempted to address these specific challenges using methods such as encapsulation.[1]

Variety of analysis techniques for the quantification of total and isolated curcuminoids in different matrices have been reported, especially spectrophotometric methods for the determination of total curcuminoids However, this approach cannot be used to quantify individual curcuminoids, so various liquid chromatographic methods have been developed for this purpose.

High-pressure liquid chromatography with UV detection (HPLC–UV) is the most common method for the determination of curcuminoids and curcumin in turmeric samples, biological samples, or dosage forms.

Due to the very labile characteristics of curcuminoids, C18 columns are preferred for HPLC analysis .[4]

HPLC (High-performance liquid chromatography or highpressure liquid chromatography) is widely applied for separations and purifications in a variety of areas including pharmaceuticals, biotechnology, environmental, polymer and food industries.

It is accomplished by injection of a small amount of liquid sample into a moving stream of liquid (called the mobile phase) that passes through a column packed with particles of the stationary phase.

The separation of a mixture into its components depends on different degrees of retention of each component in the column. HPLC is just one type of liquid chromatography, meaning the mobile phase is a liquid. Reversed-phase HPLC is the most common type of HPLC.

The reversed-phase means the mobile phase is relatively polar, and the stationary phase is relatively non-polar. HPLC instrumentation includes a Solvent reservoir, pump, injector, column, detector, and integrator or acquisition and display system.

The heart of the system is the column where separation occurs. The information that can be obtained using HPLC includes identification, quantification, and resolution of a compound. The major applications are in the area of Pharmaceuticals, food, research, manufacturing, forensics, and bio-monitoring of pollutants .[5]

INSTRUMENTATION OF HPLC The HPLC instrumentation involves pump, injector, column, detector, measuring instrument and show system. In the column the separation happens. The parts include: a) Solvent reservoir: The contents of the mobile phase are present in the glass container.

The mobile phase, also known as the solvent, in HPLC is a mixture of polar and non-polar liquid components. The polar and non-polar solvents will be changed depending on the sample composition. b) Sample Injector: The

injector might be a solitary mixture or an electronic mixture structure .an injector for a HPLC framework ought to provide infusion of the fluid specimen within the scope of zero .1 ml to one 00 ml of volume with high reliability and below air mass (up to 4000 psi). c) Pump: The pump suctions the mobile section from solvent reservoir and forces it to column so passes to detector. 42000 KPa is that the operational pressure of the pump.

This operational pressure depends on column dimensions, particle size, rate of flow and composition of mobile section. d) Columns: Columns area unit usually manufactured from clean stainless steel, area unit somewhere around fifty millimeters and three-hundred-millimeter-long and have an inward distance across of somewhere around two and five millimeters.

They're usually loaded with a stationary part with a molecule size of three to 10 millimeter. e) Detector: The HPLC detector, positioned towards completion of the column identifies the analyses as they elute from the chromatographic column.

Frequently used detectors are UV spectroscopy, fluorescence, mass spectrometric and also electrochemical identifiers. f) Data Collection Tools or Integrator: Signifies from the detector may be collected on chart recorders or digital integrators that rise and fall in many-sided high quality and also in their capability to procedure, save and also reprocess chromatographic info.

The PC coordinates the response of the indication to every component and also areas it into a chromatograph that's anything yet tough to analyze. The schematic illustration of a HPLC instrument unremarkably incorporates a sampler, pumps, and a locater.

The sampler brings the sample into the mobile part stream that conveys it into the column. The pumps convey the mobile part through the column. The detector generates a signal relative to the live of sample element rising up out

of the section, consequently taking into thought quantitative section, consequently taking into thought quantitative investigation of the instance components.

A computerized semiconductor and code management the HPLC instrument and provides data information. many models of mechanical pumps in an exceedingly HPLC instrument will mix various solvents in proportions dynamic in time, producing a synthesis slope within the moveable stage.

Many HPLC tools also have a column broiler that thinks about sterilization the temperature level at that the dividers are carried out.[6]

TYPES OF HPLC: HPLC is split into following type a) Normal phase chromatography: • Normal–phase chromatography was one of the first kinds of HPLC that chemists developed. Also known as normal-phase HPLC (NP-HPLC) this method separates analytes based on their affinity for a polar stationary surface such as silica NP-HPLC uses a non-polar, non-aqueous mobile phase (e.g. Chloroform), and works effectively for separating analytes readily soluble in non-polar solvents.

The analyte associates with and is retained by the polar stationary phase b)• Reversed phase HPLC (RP-HPLC) has a non-polar stationary phase , moderately polar mobile phase. One common stationary phase is a silica which has been surfacemodified with RMe2SiCl, where R is a straight chain alkyl group such as C18H37 or C8H17. With such stationary phases, retention time is longer for molecules which are less polar, while polar molecules elute more readily (early in the analysis).

An investigator can increase retention times by adding more water to the mobile phase c) Size-exclusion HPLC- Size-exclusion The column is incorporating with precisely controlled substrate molecules. on the distinction in molecular sizes the separation of constituents can occur. d) Ion-exchange

HPLC- The stationary part has ironically charged surface opposite to the sample charge. The mobile part used is binary compound buffer which can management pH scale and ionic strength [7].

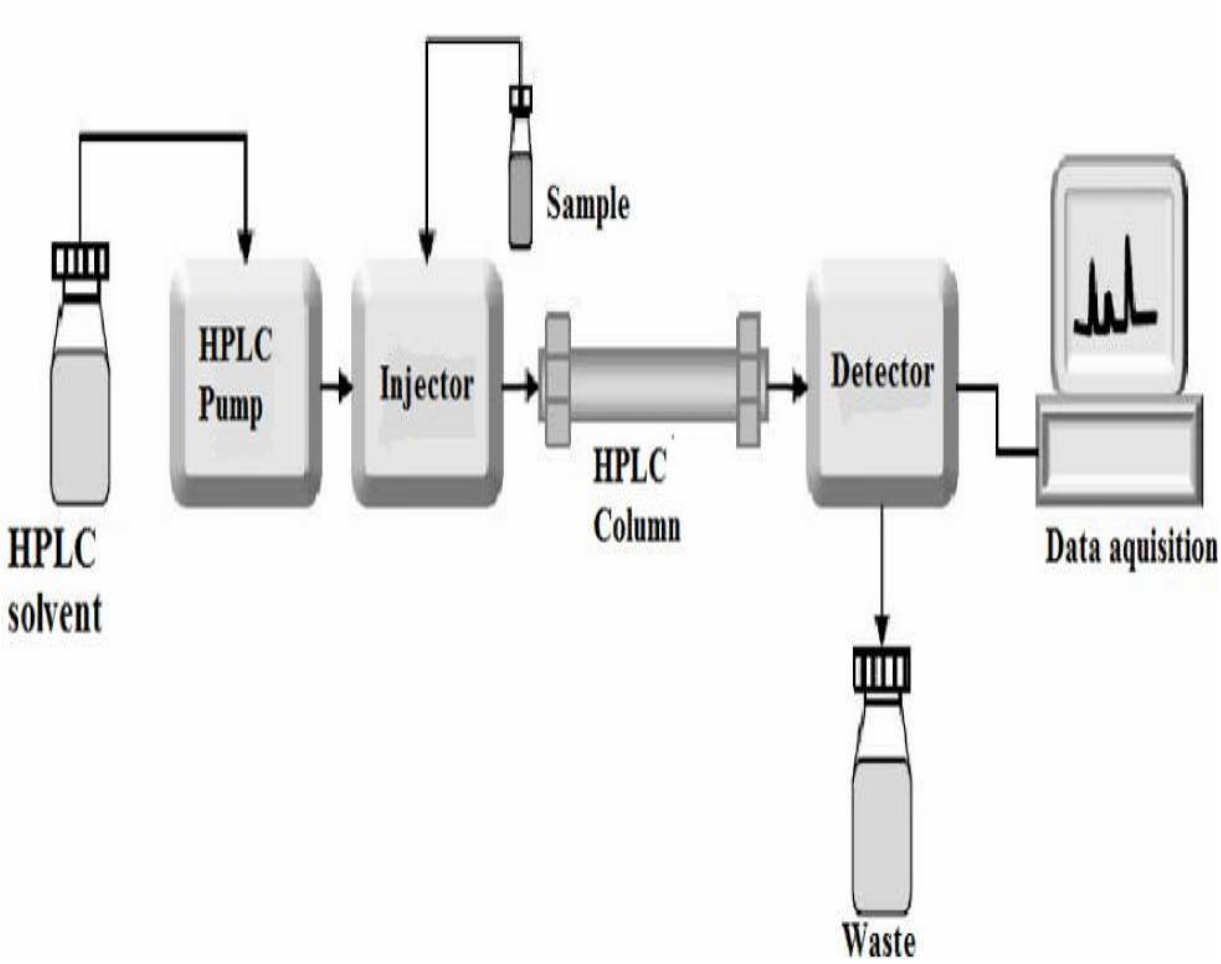


Figure 2 : HPLC device

2-Results

Figure 2.1 represents HPLC chromatogram of crude curcumin sample (methanol extracted). There are two significant peaks, the fist is single, high intensity and sharp peak at the retention time 4.4 min. the second is single, sharp but less intensity as a comparison to the first one, the retention time of this peak is 7.2 min.

The third low intensity peak at the retention time less than 10 min could be one of the compounds of the crude curcumin sample or contamination in one of the HPLC parts (injector, column, solvents, ….). This result is indicator for easy separation the two compounds as there is no overlap between them.

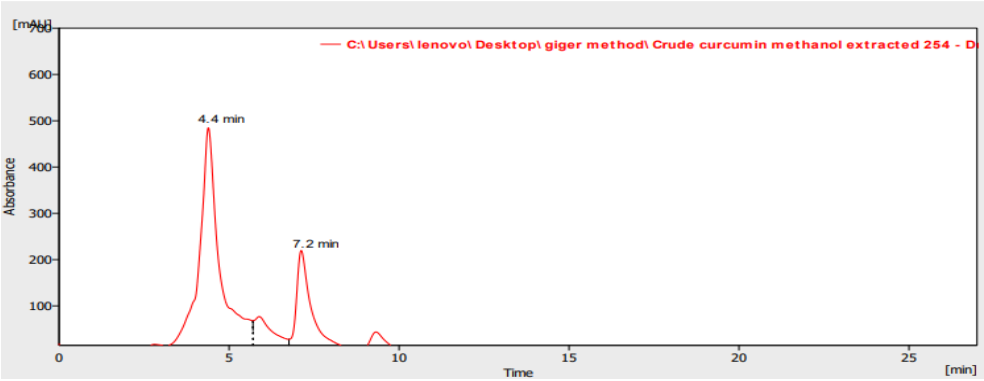


Figure 2.1: HPLC chromatogram of crude curcumin sample (methanol extracted)

Figure 2.2 shows HPLC chromatogram of purified compound at the retention time 4.4 min. The single, sharp and high intensity peak which is consistent with the peak in the figure 2.1 for the crude curcumin sample gives good signal that the used method to separate and purify of this compound was successful. In addition to that, the peak was not broad so this peak represents one compound. Moreover, the retention time of this peak is 4.4 min so it will not be loss of the solvents during collection of the peak as the retention time is not far away.

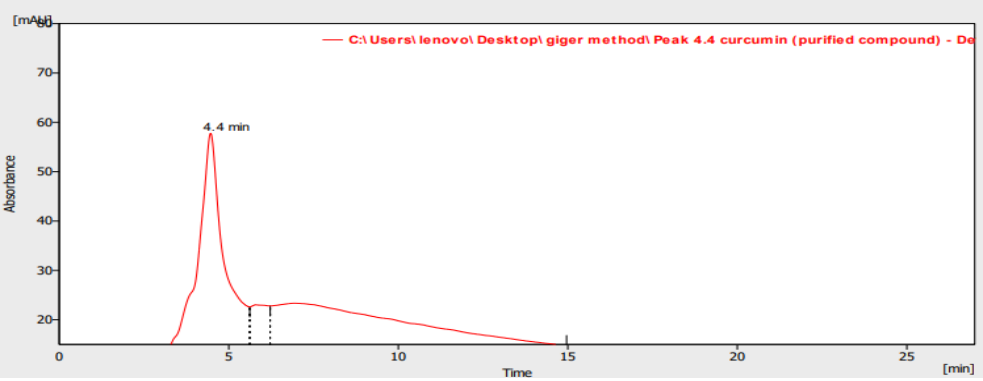


Figure 2.2: HPLC chromatogram of purified compound at the retention time 4.4 min

Figure 2.3 clarifies HPLC chromatogram of purified compound at the retention time 7.2 min. the used method to separate of this compound was successful as in this Figure (2.3), there is single, sharp, high intensity and narrow peak (the starting to ending of the peak is less than one min).

According to this result, the process of the collection and purification of this compound is by hand. The other very low intensity peaks at the retention time less than the target peak are could be contaminations or some of the compounds of the crude curcumin sample were separated but the used method was not suitable for this compounds.

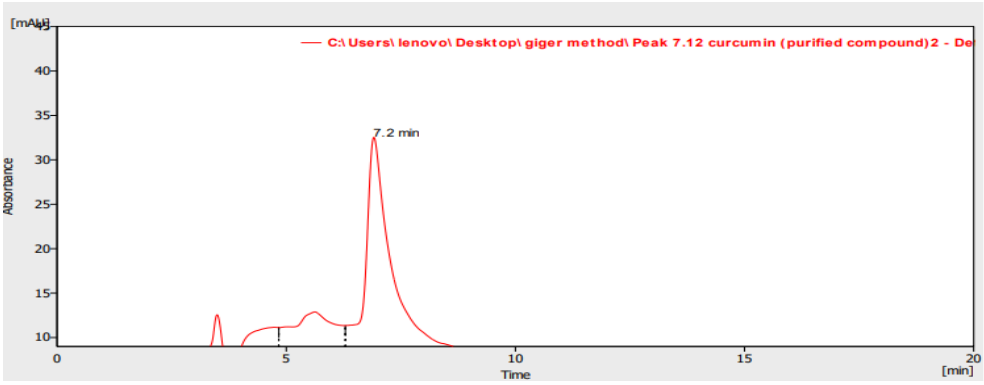


Figure 2.3: HPLC chromatogram of purified compound at the retention time 7.2 min

3-Experimental

Analytical methods RP−HPLC

HPLC analysis was performed by a the sykam HPLC S 600,GermanyRP−C18E, 4.6 × 250mm , a 5µL coulum (Germany), injection 10 uL and a flow rate of0.7 ml min−1. The detector wavelength was set at 220 nm. The column oven temperature was set at 30 °C. Unless stated otherwise, solvent A was 5% H2O with 95% acetonitrile and add formic acid 0.1% add to the solvents .

Procedure :

1- 250 mg of curcumin powder was grind .

2- 10 mg of curcumin powder was weight and dissolved with methanol

3- The solution was mixed well by the vortex.

4- The sample was sonicated by sonicater for 3 minutes then mixed again by vortex .

5- The sample was centrifuged for 3 minutes then 1 ml of the clear solution was moved from eppendrof vial to HPLC vial to be ready for run for HPLC .

6- From result of HPLC 2 purified soluble compounds was collected each alone with certain volume.

7- The compound was put in the freezer for 24 hour then collected with the freez dryer.

8- 1 mg of the purified compound was weight and dissolved by methanol and checked by HPLC.

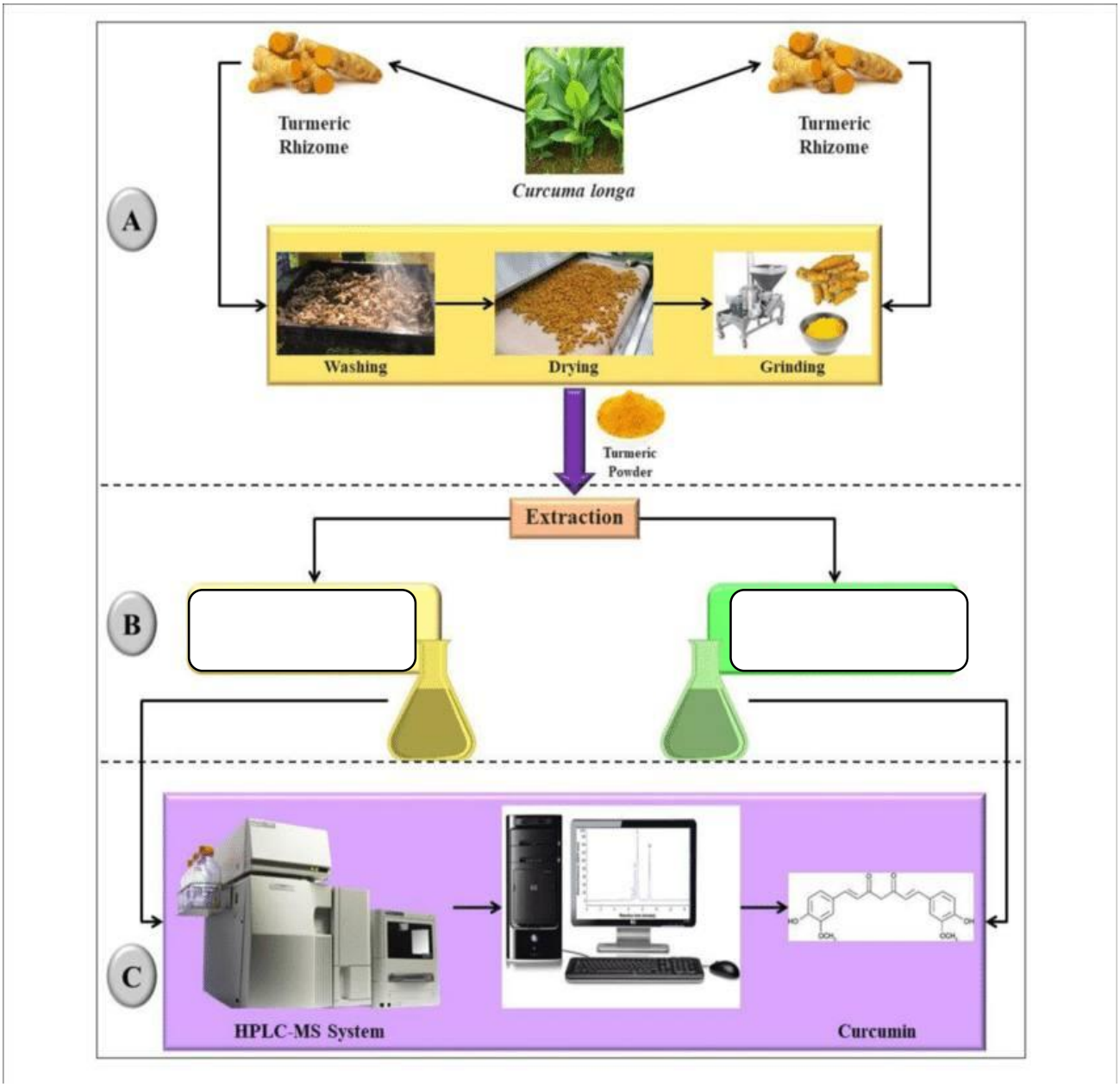
	Gradient Table					
	Time (min.)	**A (%)**	**B (%)**	**C (%)**	**D (%)**	**Flow (ml/min)**
1	**Initial**	**5.0**	**95.0**	**0.0**	**0.0**	**0.700**
2	**15.00**	**20.0**	**80.0**	**0.0**	**0.0**	**0.700**

Table 3.1 : It represents a method of separation and purification of compounds

4. conclusion

1. Two compounds were separated successfully. The used method was suitable for getting a high purified compound for each one .

2. There is the ability to get more than one high purified compound from alcoholic extract for curcumin



5 - Refrences

1_Tian Jianga, Raja Ghoshb, Catherine Charcosseta. Extraction, urification and applications of curcumin from plant materials-A comprehensive review. published by Elsevier,2021 :1_42.

2_Gökdemir, B., Baylan, N., & Çehreli, S. Application of a Novel Ionic Liquid as an Alternative Green Solvent for the Extraction of Curcumin from Turmeric with Response Surface Methodology: Determination and Optimization Study. February(2020) :1–11.

3_Imran Ali, Ashanul Haque, Kishwar Saleem1. Separation and Identification of Curcuminoids in Turmeric Powder by HPLC Using Phenyl Column. Royal society of chemistry (analytical method) :1_32.

4_panelBruno Fonseca-Santos, Maria Palmira Daflon Gremião, Marlus Chorilli . A simple reversed phase high-performance liquid chromatography (HPLC) method for determination of in situ gelling curcumin-loaded liquid crystals in in vitro performance tests . Arabian Journal of Chemistry Issue 7, Volume 10 ,November 2017 :1029-1037.

5_Abdu Hussen Ali .High-Performance Liquid Chromatography (HPLC).

advancechemjournal ,June 20, 2022: 1_11.

6_Pooja S Murkute, Paresh H Patil, Dr.Gajanan S Sananp, Nakul P Kathar, Aishwarya P Pimple. A REVIEW ON HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) 2022 IJRTI | Volume 7, Issue 2 : 1_7.

7_ Natashya Evangeline Fiorelia, Anthony Dharma Wibowo, Natanael Leonard Lae, Allison Ang, Oki Krisbianto. Types of High-Performance Liquid Chromatography (HPLC) Columns .Jurnal Teknologi Pangan, Vol. 5 (2022): 1-1