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Centrifugally accelerated thin layer chromatography for isolation of marker compounds and bioactives

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Abstract
Medicinal plants containing essential bioactive ingredients are used to cure disease or disorder since time immortal. One of the aims of medicinal plant research is the isolation and identification of markers/ bioactive compounds. Isolation of the markers compounds and bioactive plant constituents has always been a challenging task for the researchers. Separation of these components from the medicinal plants includes the use of combination of chromatographic techniques such as column chromatography, preparative thin layer chromatography, preparative high performance liquid chromatography, droplet counter current chromatography, centrifugal thin layer chromatography, etc. Centrifugal thin layer chromatography which makes use of centrifugal force for separation of multi-component system offers extensive platform for the isolation of phytoconstituents from medicinal plants. This review focuses on basic principle, instrumentation and advantages of centrifugal thin layer chromatography. Additionally, this article also highlights on various applications of this technique for isolation of plant molecules.

Keywords: Bioactive, centrifugal TLC, isolation, marker compounds

1. Introduction
Medicinal plants are rich in secondary metabolites and have been identified for therapeutic purposes. Recent estimates indicate that about 80 % of people in developing countries still rely on herbal medicines for their primary health. However, the use of plant based medicines is not restricted to developing countries; these medicines are also receiving recognition in the developed countries. The use of alternative medicines comes in the picture when allopathic medicine fails to treat the disease. Additionally, herbal medicines are of being natural origins are considered to be safe and not toxic [1].

Despite of numerous advantages of herbal medicines over conventional medicines, their worldwide acceptance remains incomplete because of lack of proper standardization techniques. Marker based standardization of plant based medicine has become more popular nowadays which involves identification of key and unique phytoconstituent/s in plant/extracts/formulation as markers and development of suitable analytical method for monitoring them [2].

2. Marker compounds
Marker compounds or chemical markers are chemically defined plant molecules which may or may not possess therapeutic activity but can be utilized for quality control of herbs [3].

According to Hongxi Xu *et al.,* there are eight categories of chemical markers namely therapeutic components, bioactive components, synergistic components, characteristic components, main components, correlative components, toxic components and general components used with fingerprint spectrum [4].

3. Bioactives [5, 6]
Markers with therapeutic activities are known as bioactives. They are plant metabolites synthesized for self-defense and other purposes and have the potential to be used by humans for a variety of applications. Thus, the search for therapeutic agents and bioactive compounds to fight against emerging and existing diseases is ongoing process. Since bioactive markers are responsible for therapeutic activity, they are given more importance rather than chemical markers. The typical bioactive compounds are produced as secondary metabolites. Thus, bioactive compounds can be defined as secondary plant metabolites in the form of glycosides, tannins, terpenoids, flavonoids, phytosterols or alkaloids producing pharmacological or toxicological effects in man and animals [7, 8].

4. Isolation of marker compounds/bioactives [9]

Isolation of constituents from plants with highest purity is a long and tedious process and requires expertise and profound knowledge of phytochemistry and separation chemistry [10].There are a number of techniques available for isolation of marker compounds or bioactives from medicinal plants such as preparative thin layer chromatography (PTLC), preparative HPLC, droplet counter current chromatography (DCCC), centrifugally accelerated thin layer chromatography (CTLC), etc [11].

CTLC is a preparative chromatographic technique where centrifugal force is used for separation of multi-component system. It is a preparative, centrifugally accelerated, radial thin-layer chromatographic technique. It allows rapid separations using centrifugal action of the spinning rotor driving the mobile phase through the adsorbent layer. Thus, CTLC offers a widespread platform for the fractionation, separation and purification of plant molecules.

5. Centrifugal Thin Layer Chromatography

5.1 Principle [11]

The centrifugal force is generated by spinning of the support disk and the mobile phase is applied at constant flow rate. As the mobile phase elutes, it carries the sample along with it thereby creating spherical bands of the separated components. The separated components are then swirled off from the edge of the rotor together with the mobile phase.

5.2 Parameters to be considered in centrifugal TLC [12]

There are two factors that are considered in centrifugal TLC: flow rate of mobile phase and rate of rotation of the support.

Flow rate of mobile phase

Along with the resolution, separation time is dependent on the flow rate of mobile phase. High flow rates speed up the separation process but requires larger volume of solvents.

Speed of rotation

The elution rate as well as the separation factor is dependent on the rate of rotation of the chromatographic system. The maximum separation / resolution is attained at low rates of rotation of chromatographic system.

Highest resolution can be obtained at medium flow rate and rotation speed.

6. Instrumentation of centrifugal TLC [13-16]

The instruments used for centrifugally accelerated device used for performing preparative thin layer radial separations are known as Chromatotron [13] and Cyclograph System [14].

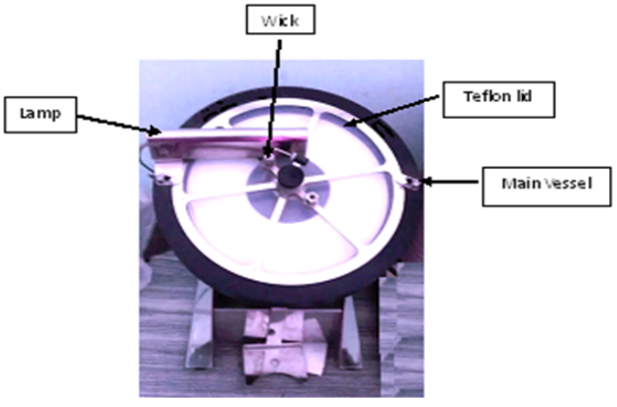

Fig 1: Image of cyclograph

The stationary phase used is sorbent coated as thin layer on a rotor which is driven by motor. The sample solution to be separated is applied on the rotor via the inlet and wick. When elution is carried out using mobile phase, concentric bands of separated substances are formed. As development progresses, the concentric bands of separated substances come to the edge of the rotor and leave the rotor. The channel collects the eluate and brings it to the output tube [15].

Various instrumentation components include main vessel, solvent pump, rotor, Teflon lid, wick and collection vessel.

6.1 Main vessel

The main vessel is made up from acetal polymer. After the chromatography is complete, the solvent that soften or swell acetal has to be washed out with hexane. Acetic acid, trimethyl amine and ammonia can be used as mobile phase additives. Damage to plastic parts of vessel can be reduced by passing nitrogen.

6.2 Solvent pump

The flow of mobile phase can be regulated by pump. In addition, it can be used for introduction of sample solution.

The flow rate of mobile phase can be varied for different thickness of stationary phase as given in table 1.

Table 1: The flow rate at different sorbent thickness

Thickness of Sorbent Layer (mm)	Flow Rate (ml/min)
1	2-3
2	4-6
4	8-10
6 & 8	10-15

6.3 Rotor

The stationary phase comprising of circular disc coated with sorbent layer is called as a rotor. The rotor has a hole in the centre so that it could be fixed on the motor of the instrument. Rotors with various thickness of sorbent layer are available and based on thickness of sorbent layer; the amount of sample to be loaded varies (Table 2).

Table 2: Sample load for different sorbent thickness.

Sorbent Layer Thickness (mm)	Sample Load (mg)
1	250
2	750
4	1500
6 & 8	2500-4000

6.4 Teflon lid

A UV transparent Teflon lid is covered on main vessel which allows user to keep an eye on moving band.

6.5 Wick

The main function of the wick is to carry the sample solution to the rotor. The wick is generally made up of thin fluffy polyester string. Sewing cotton or threads are not recommended.

6.6 Collection vessel

A specially designed collection channel across the periphery of the rotor collects the eluting mobile phase. As the main vessel is mounted slightly tilted, the gravity plays role in draining of eluent.

7. Chromatographic parameters in CTLC

7.1 Stationary phase (Sorbent)

The various sorbents used are Silica gel (TLC standard grade with gypsum), acidified silica gel, aluminium oxide GF- 254, silica gel containing silver nitrate and gypsum. Along with sorbent, calcium sulphate hemihydrate is frequently used as binder.

7.2 Mobile phase

The selection of mobile phase depends upon the sample to be separated. Mostly the solvent system giving a low Rf (i.e. 0.2-0.4) are preferred. The solvent used for mobile phase system can range from hexane to methanol. The chromatograph is compatible with all common chromatography solvents, including acetic acid but is not compatible with mineral acids. Gradient elution is generally preferred.

7.3 Sample application

The sorbent layer should be entirely saturated and equilibrated with mobile phase prior to sample application. The sample to be separated (1 to 2 mg) should be dissolved in 0.5- 2 ml of solvent and filtered. The sample solution thus prepared can be applied on sorbent.

7.4 Detection

For detection of bands, UV lamp is placed over the Teflon lid of the instrument. Detection is generally done at short wavelength (254 nm). Compounds that do not absorb UV rays can be analyzed by conventional TLC after collection.

8. Advantages of CTLC [11, 17]

8.1 Rapid separation: Since the development is centrifugally accelerated, quick separation can be achieved generally within 20 minutes. For unstable compounds, the separation time can be reduced to about 5 minutes.

8.2 No scraping of bands/ spotting of sample: Solution of the mixture to be separated (sample) is pumped into the chromatotron. So, there is no need to spot the sample as in conventional TLC. The product fractions that are separated are released in the form of solution. So, no need of scrapping the bands as in conventional TLC.

8.3 Easier gradient elution: Easy to perform gradient elution. The inlet of the pump can be changed from a weak polar solvent to a more polar solvent during the run. This facilitates extensive range of polarities to be used. It allows the rapid separation of complex samples like natural products.

8.4 No requirement of UV monitor or recorder: A 25 cm diameter quartz lid allows direct observation of UV absorbing compounds during the separation. We can monitor the separation as they happen with the built in UV 254 lamps. Optionally, a UV 366 source can also be used to detect fluorescent compounds.

8.5 No oxidation of compound: Provision of nitrogen purging can prevent oxidation of compounds.

8.6 Reusable rotors: Rotors can be reusable. Mostly begin the run with low polarity mobile phase and then finish it with strongly polar solvent. This allows the strongly adsorbed compounds to be cleared from the rotor. This would enable the rotor to be reused for other samples too.

8.7 High capacity rotors: Availability of rotors with varying thickness allow loading of sample ranging from milligram to gram.

8.8 Compactness: The chromatotron is compact and thus can moved from one lab to another lab easily.

9. Disadvantage of CTLC

9.1 Costly: It is very expensive i.e it costs $ 3,500 for gravity feed version & $ 4,300 for peristaltic pump version.

10. Applications of CTLC

Centrifugal TLC is widely used for the isolation and fractionation of alkaloids, glycosides, anti-oxidants, enzyme inhibitors, HIV inhibitors as well as vitamin D from medicinal plants.

1. Dentatin has been isolated from *Clausena excavate* using TLC aluminum sheets precoated with silica gel 60 F254 as sorbent and elution was carried out using petroleum ether and dichloromethane mixtures of increasing polarity by CTLC [18].
2. Four triterpenes lupeol, campesterol, stigmasterol, β-sitosterol; a sesquiterpene, β-selinenol and two alkaloids, liriodenine and lanuginosine were isolated from *Meiogyne monosperma* by CTLC [19].
3. A dipeptide namely aurantiamide benzoate and two xanthones, cycloartobiloxanthone and dihydroartoindonesianin C were isolated from the stem bark of *Artocarpus kemando* by chromatotron using hexane: acetone (13:2, v/v) as eluents [20].
4. Isolation of flavonoid fraction from the leaves of *Ricinus communis* was carried out by CTLC using silica gel as stationary phase and n-hexane: methylacetate: methanol mixtures as mobile phase [21].
5. Various subfractions of triterpenoids were isolated from *Garcinia prainiana* on chromatotron using silica gel 60 PF254 as stationary phase and mixture of hexane and ethyl acetate in increasing polarity as mobile phase [22].
6. Bioactivity guided fractionation was carried out on CPTLC (1 mm silica rotors) using n-hexane: acetone (99.5: 0.5, v/v) as eluent to yield two potent diterpenes i.e 6-deoxytaxodione and taxodione from *Cupressus sempervirens* [23].
7. Various bioactives like emodin-8-glycoside and rutin were isolated from *Muehlenbeckia hastulata* using silica gel 60 PF254 as stationary phase and ethyl acetate: methanol (96: 4, v/v) as mobile phase [24].
8. Purification of saponin hydrolysates was carried out using CTLC. A mixture of chloroform: methanol (99.5:0.5, v/v) was used as mobile phase for oleanolic acid, whereas chloroform: methanol (98:2, v/v) was used for hederagenin [25].
9. Isolation of alkaloids which is mainly a mixture of three C_{19}-diterpenoid alkaloids, viz., aconitine, 3-deoxyaconitine, and mesaconitine was done from crystalline *Aconitine merck* using gradient elution on a rotor coated with mixture of aluminium oxide 60 GF_{-254} and calcium sulphate hemihydrate. The mobile phase used was hexane: diethyl ether (25: 75, v/v) for 3-deoxyaconitine, diethyl ether: methanol (99.9: 0.1, v/v) for aconitine and methanol: diethyl ether (1.5: 98.5, v/v) for mesaconitine [25].
10. Separation of bis-diterpenoid alkaloids (delphinine and 14-acetyldelcosine) from the seeds of *Delphinium*

staphisagria was carried out using alumina rotor (1mm) and elution was carried out using diethylether and increasing percentage of methanol [26].

11. Isolation of new triterpenic glycosides present in *Passiflora quadrangularis* was carried out using silica gel as sorbent and ethyl acetate: ethanol: water (16:3:2, v/v/v) as eluent by CTLC [27].
12. Separation of diastereoisomeric 2-arylpropionic acid derivatives was done using silica gel 60 GF254 as sorbent and chloroform: cyclohexane: tetrahydrofuran (54.2:45:0.8, v/v/v) as eluent by CTLC [28].
13. Alkaloids (isorhoeadine and papaverrubine) from *Papaver rhoeas* were purified using aluminium oxide plates with cyclohexane: toluene: diethylamine (320:80: 1, v/v/v) as eluting solvent and separation of quinidine and quinine was done using cyclohexane: chloroform: diethylamine (30:9:2, v/v/v) at a flow-rate of 0.5 ml/min. Also the separation of alstonine, ajmalin and rescinnamine was carried out on aluminium oxide plates using cyclohexane: toluene: diethylamine (35:1:3, v/v/v) as mobile phase at a flow rate of 1.5 ml/min [29].

11. Discussion

CTLC covers the advantages of both preparative TLC and column chromatography and provides fast preparative separations. Cyclograph is simple and compact as compared to HPLC chromatograph. This technique can offer better option over tedious preparative TLC and conventional chromatography for fractionation, isolation and purification purpose.

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